

Does moratorium mean a test-ban?

President Bill Clinton is with the angels in extending the suspension of nuclear tests. The hope now is that a test-ban will follow. But the model of 1979 is not a good model.

PRESIDENT Bill Clinton deserves credit for having declared last week that the United States will not test nuclear weapons during the coming fifteen months provided other nuclear powers follow suit. Although he may have had little choice in the matter, not all his generals will have shared his view. He will also have had to counter the dismay of friendly governments such as the British and French. (The British government believes it needs access to Nevada for testing warheads for the missiles it is now developing for its four Trident submarines; the French government, whose conception of the utility of nuclear weapons is more closely linked with the need for self-defence, fears that Clinton's example will be unduly constraining.) But, in the end, they will go along with Clinton's wish. The big question is whether the other nuclear powers will fall in line.

Which are they? That is not the simple question it once was. China is a nuclear power. Russia is certainly another, but President Boris Yeltsin has other complicated issues on his mind; this may not be a time for demanding from his armed forces a concession they would regard as another sacrifice. Then what of Kazakhstan, Byelorussia and the Ukraine, and the ownership of the once-Soviet missiles stranded on their newly independent territory? Only last week, the parliament of the Ukraine (a somewhat unreliable indicator) was proclaiming that the Ukraine is now an independent nuclear power. The governments of the ex-Soviet republics are in such disarray that even if they said they would fall in with Clinton's moratorium, their word would carry little weight even for fifteen months. Then there is a substantial and growing group of crypto-nuclear powers, from Israel to North Korea. Where will they fit in — and with what?

First step

The best answer is that the moratorium should be made the first step towards a comprehensive ban on the testing of nuclear weapons. When it expires in September next year, there will be only nine months to go to the opening of the review conference of the Nuclear Non-Proliferation Treaty (NPT). And that conference may be the last in the series; its members (now more than 120 strong) will be required then to make a positive declaration in favour of its continuation. Their traditional complaint is that the major nuclear powers have not done enough to arrange for substantial measures of arms control. At the last chaotic review conference in 1990, nobody had reason to expect that the Soviet Union would fall

apart in quite the way it did. Since then, the United States and the rump of the Soviet Union have done wonders to arrange restrictions on the disposition of nuclear arms in Europe and treaties have also been signed on strategic nuclear weapons (and the destruction of those considered surplus to requirements). In earlier circumstances, that would have been enough to persuade the members of the treaty to continue with it. But the new uncertainties, created by the likes of North Korea and Iraq as well as the Ukraine, argue for further concessions to the non-nuclear members of the NPT.

A comprehensive test-ban would excellently do the trick. It would be a more secure promise than any other that the established nuclear powers have at last turned their backs on the further improvement of their nuclear weapons. Together with the unratified treaties on strategic arms, that would be a sign that what once seemed the inexorable sophistication of nuclear weapons had been halted. It may even be that Clinton has something like this in mind. Fifteen months should be just long enough for the major players to negotiate the terms of a treaty. Mr Warren Christopher, the US Secretary of State, who was an assistant secretary at the State Department when the Carter presidency had all but secured agreement on a test-ban by the second half of 1979, may have already dusted off the documents of that time.

Multilateral

Sadly, the old documents will not serve the present purpose. The world was a simpler place in 1979. Then, the proposed treaty would have bound only its three depository signatories — Britain, the Soviet Union and the United States. Now, it will have to be a multilateral treaty, including all members of the NPT. Then, the ostensible stumbling blocks concerned the numbers of monitoring sites required to make sure that nuclear powers signing the treaty did not cheat; those objections were generally understood to be placeholders for the political will to strike a deal, which never materialized. Now, the problem is to arrange that even if a state has not acceded to the treaty, it will run the most dreadful risks if it conducts a nuclear test. That is why even fifteen months will hardly be enough in which to define the terms of a present-day comprehensive test-ban treaty. Let us hope that Clinton's people see the urgency of the problem and tackle it energetically.

Inevitably, the process will not be free from trouble.



The British government's attachment to nuclear weapons is an illustration of the difficulties. Britain is now a nuclear power because of the Ottawa agreement of 1943 (signed before President Clinton was born) by which Britain, the United States and Canada agreed to share the nuclear technology then in prospect. That is the basis on which Mr Harold Macmillan persuaded President John Kennedy in 1962 that Britain should be supplied with the air-launched 'Skybolt' missiles being developed for the US Air Force; when Skybolt failed, Britain was allowed to build Polaris submarines instead. Now it has traded up to Trident, but will assemble the warheads for their rockets with its own resources. At no stage in the past decade has this expensive course of action been questioned seriously except by a few of the people best qualified to know, and notably by the late Lord Zuckerman (see *Nature* 361, 392–396; 1993).

A curious situation has thus arisen. The British government, seeking to cut its defence spending to match its revenue, has fought tooth and nail for the amalgamation of ancient army regiments, but has never asked itself why it is still in the military nuclear business. Its chief political opponents, still smarting from the electoral damage they did themselves as recently as the early 1980s by advocating unilateral nuclear disarmament, seem now incapable of acknowledging that circumstances have changed. Britain has become that contradiction in terms, a military nuclear power by tradition. Denial of access to the Nevada testing sites for the next fifteen months may in the circumstances be just what is needed to put that neglected issue on the political agenda.

The realism required of Britain by self-interest is required of others in the international interest. France can justify its nuclear weapons chiefly by reference to drastically changed circumstances that undo the upheavals of the past few years. Even so, a pooling of strategic interests with Britain would benefit both. China is a more important influence; the need, which Clinton should brood about before renewing China's trade advantages a year from now, is that China should be prepared to follow Gorbachev's Soviet Union in its willingness to negotiate on nuclear weapons. Then it would be possible to tell the NPT review conference a compelling tale. That is the prize behind Clinton's moratorium. And that is a necessary but not sufficient condition for avoiding the spread of nuclear weapons. □

Psychiatric care

Britain's scheme for caring for as many as possible of its indigent population has made a poor start.

IN the bad old days, it was commonplace that people suffering from schizophrenia and other disabling psychiatric diseases would be incarcerated once their disability had been established and diagnosed. The usual

explanation, from early in the nineteenth century, has been that there was no alternative for people with severe illness. But then, of course, it also emerged that people locked up for years on end would lose their capability to live outside the hospitals to which they were sent. 'Institutionalization' is the word for that phenomenon. The accumulation of the minor indignities of the psychiatric way of life not unnaturally saps a person's confidence in him or herself.

That is the reason why, in the late 1950s and early in the succeeding decade, the advent of drug treatment for psychiatric illness seemed such a boon. People suffering from clinical depression could suddenly be given drugs to lift their mood, those with schizophrenia one or other version of the phenothiazines, while even manic-depressive illness could be treated by, astonishingly, ionic lithium. For all the good reasons, in Britain and many other countries, people started dreaming of the time when the psychiatric hospitals would be emptied of their chronic patients who would, with the aid of drugs, be returned to the communities from which they had come. But it was naturally acknowledged from the outset that people with a history of psychiatric illness could not simply be emptied onto the streets and allowed to fend for themselves, whence there sprang in Britain the concept of 'Care in the Community' — an arrangement by which local authorities were given extra funds (transferred from the health services) and responsibility for caring for the indigent and chronically sick in their areas.

Although the scheme has been talked of in Britain for a quarter of a century, it is only now coming into effect. And the signs are that it is not functioning as intended. Local authorities are required to provide care for all who need it, and being relatively unskilled at caring for the psychiatrically disabled, tend to concentrate their attention on those deserving people who are the more easily biddable. Discharged psychiatric patients tend to be offered the short straws; they are, after all, awkward customers, and they rarely offer thanks for the attention paid to them.

It may well be, of course, that it is too soon in the administration of an ambitious and unprecedented programme to make a final judgement, but the numbers of psychiatrically disabled people roaming Britain's major cities are a sombre reminder that care in the community will have to do a lot better before it can be said to have succeeded. The signs are that there is too little money overall, and too few facilities for the spells of asylum that all but the most marginal cases of psychiatric illness need from time to time, when the Department of Health in Britain is eagerly planning to let users determine the pattern of its research (see page opposite), there is a case for asking that some of the funds available should go on a search for more effective drugs, for more effective ways of caring for newly discharged patients and for more effective ways of breaking the vicious cycle of disappointment and despair. □

Driving UK health research by responding to users' needs

London. Health managers know — or should know — best. That is the main thrust of a strategy for research and development (R&D) in Britain's National Health Service (NHS) given an extra push last week by Mrs Virginia Bottomley, the health secretary.

Bottomley says the Department of Health intends to use the £60 million a year it spends on R&D to help create an "evaluative culture", enabling regional health managers to make the most cost-effective use of advances in science and medicine. More bluntly, health research is now one means of achieving the government's goal of increased efficiency in the health service.

To this end, funding will be focused on tasks such as assessing and broadcasting the effectiveness of new health technology, ranging from key-hole surgery to gene therapy. Funds will also be targeted on research needs identified by clinicians and health managers. More controversially, lower priority will be given to projects defined primarily by the research community.

The new strategy follows criticism three years ago by the House of Lords select committee on science and technology that the NHS lacks a coherent approach to biomedical research. It also reflects two government priorities: keeping a lid on public spending, and achieving better value for money in spending health service funds.

Combining these goals has been the prickly task of Michael Peckham, formerly head of the British Postgraduate Medical Federation in Hammersmith, who was appointed two years ago as the health department's first director of R&D (a post whose creation was a central recommendation of the House of Lords committee).

In its initial response to the Lords report in 1991, the health department signalled its readiness to give up being a passive recipient of new technology, and to establish a coherent R&D programme (*Nature* 351, 7; 1991). A new report, published last week as *Research for Health*, describes progress so far and several fresh initiatives.

The main message is that the government remains committed to letting managers and users, not investigators, shape the pattern of health research in Britain. The new R&D programme seeks to achieve this by integrating R&D into the management and practice of the NHS.

Multidisciplinary groups of clinicians and health managers have already been set up in some fields to draw up short-lists of research priorities, which will subsequently be funded through regional health authorities. Two reviews have already been completed — in mental health and cardio-

vascular disease; there are others in topics from cancer to dentistry.

A new centre, the Cochrane Centre, has been set up in Oxford to help researchers design and evaluate clinical trials. And a standing group on health technology was



Michael Peckham: charged with getting value for (less) money.

created in February to help the NHS identify what technology it should promote.

Support for university-based research is to be concentrated on three or four multi-programme centres. Proposals are already being received for the first, on primary health care; Peckham announced last week that the department is soon to invite proposals for a second, covering research-based clinical medicine.

There is also to be a national R&D information network, to be completed by the end of the year as a tool for health managers. Central to the network will be one or two co-ordinating centres, for which bids have also been requested, to develop new techniques for collating and disseminating information on recent research.

Many elements of the new strategy have received a broad welcome from the research community. But some also worry about their implementation — as well as the commitment to cost-saving that underpins them.

Although concentrating research funds on large multidisciplinary centres may bring increased value for money, by forcing other research groups into the competitive world of contract research, it risks undermining

the strengths and long-term perspectives of smaller specialist research units.

"The new strategy could reduce the science-push from groups outside the main centres, even though experience has shown that these groups often come up with the most novel ideas" says Bleddyn Davies, professor of social services at the University of Kent, and chairman of the Association of Department of Health Funded Researchers.

Ken Judge, director of the King's Fund Institute in London, warns that the government's concentration on cost-effectiveness in the health service has distracted attention from other criteria for allocating both services and research funds, particularly equitable access by patients.

Finally, the question of money. Although the department has promised to increase research funding from 1.0 to 1.5 per cent of its overall budget, few details have yet been published of what extra funds will be available — or where they will come from.

Researchers are justified in feeling nervous. Ironically, the spirit of the seventeenth century physician William Harvey is being invoked to justify the importance of linking fundamental and clinical research — precisely at a time when Harvey's own institute, St Bartholomew's Hospital, appears to be heading for oblivion, a victim of the combined pressures of rationalization and cost-cutting in London's medical services.

David Dickson

Selling science

London. William Waldegrave, Britain's science minister, announced last week that the government is to provide grants worth £100,000 from the budget of the Office of Science and Technology to support projects aimed at improving the public understanding of science.

The new scheme delivers a promise in the recent White Paper to promote a greater awareness of science. It is intended to complement efforts by outside organizations such as the Wellcome Trust and the Gatsby Foundation, which already runs a major programme designed to raise knowledge about technology among school children.

"Projects can range from science events to new information databases to science-by-mail schemes," Waldegrave told a meeting of the Association of British Science Writers. "We have an open mind." He also said that one of the government's aims in launching the scheme is to find out more about the relative effectiveness of different approaches and strategies.

D.D.

Barrage of mud fails to stick on sinking Super Collider

Washington. A nine-hour interrogation of the management of the Superconducting Super Collider (SSC) by a congressional subcommittee last week failed to prove that funds have been mismanaged, let alone misappropriated, by project contractors.

The SSC management was defending itself against allegations that it spent \$203 million imprudently and that it wasted a much smaller sum — less than \$1 million in total — on unnecessary items, ranging from pot plants to “souvenir items, flowers, donuts and coffee.”

The source of the allegations is an internal audit at the Department of Energy (DoE) whose essence was leaked to the public two days before the crucial vote in which the SSC was killed off in the House of Representatives (see *Nature* 364, 659; 1993). The publicity that followed undoubtedly damaged the SSC in the crucial vote.

During last Wednesday's marathon session of the oversight subcommittee of the House Commerce committee, chaired by John Dingell (Democrat, Michigan), managers of the project lead contractor, Universities Research Association (URA), insisted that the \$8.2 billion project was substantively on schedule and within budget. General manager Ed Siskin said that the project is now 20 per cent complete, but that only 4 per cent of the \$840 million contingency fund had so far been used, and that unbudgeted costs now foreseen would consume only 12 per cent of that sum.

The bulk of the \$203 million referred to cost overruns arising from engineering changes in subcontracts, and to subcontracts granted to specialist DoE laboratories without competitive tender. But no evidence was presented that the engineering changes were avoidable, or that another cheaper source could have done the work. Auditors from DoE and the General Accounting Office (GAO) thus could not say how much of the \$203 million was actually wasted.

Dr John Toll, president of URA, said the work was done at the DoE laboratories because “that is where the expertise exists to build superconducting magnets.”

What of the pot-plants, widely jumped on as the outstanding single example of SSC profligacy? Paul Gilbert, project director of the main architecture, engineering and construction subcontractor PB/MK, defended the \$2,000–\$3,000 a month spent on leasing them as “a good management decision” following representations by a staff committee on behalf of 400 design engineers required to work in warehouse space the use of which, Gilbert said, was saving \$500,000 annually against the

cost of local office space.

But Energy Secretary Hazel O'Leary is unconvinced. She told the panel that she accepted the criticisms of URA as lead contractor. “The attitudes of the leadership of the laboratory are simply unacceptable to this administration, to my department and to me personally,” she said.

O'Leary promised to review URA's contract within 30 days, and choose between termination, renegotiation and splitting into two, with URA retaining responsibility for science and a new contractor taking on construction. But when pressed, she declined to testify that the project was either over budget or behind schedule, stating only that it “has been reported to me”.

According to one very senior congressional aid, O'Leary — who is believed to be personally unenthusiastic about the SSC despite her public support for it — is going along with the allegations of mismanagement in the forlorn hope that a “new, reformed” SSC will stand a better chance of winning support in the Senate when it considers the matter in September.

Defending his university consortium

patiently, but with little apparent passion, Toll denied that URA, originally founded to build the Fermi National Accelerator in Illinois, was unfit to manage the construction side of the project. “We have always had someone on our board with real construction experience”, he said, listing chief executives of major building firms who served on it.

Cross-examined at length about an accounting change this spring, which introduced a \$70 million ‘negative management contingency’ to cover expected extra costs, Ed Siskin said that DoE had always been informed of all such costs, on a monthly basis. “There seems to be a feeling that we tried to hide these things, but all we did was to try to advertise what was going on and what would happen in the future”, he said.

Victor Rezendes of the GAO told the subcommittee that URA documents identify potential cost overruns on SSC of \$2 billion, of which energy department and URA officials consider \$600 million, or three-quarters of the contingency fund, are “likely” to materialize.

But stretching the project by three years to placate its critics will cost at least \$1.6 billion, Rezendes says, and foreign contributions will fall short by \$1.3 billion — the costs of Congressional prevarication. By that yardstick, URA's managerial shortcomings, such as they are, seem less than relevant to the SSC's current difficulties.

Colin Macilwain

Europe backs ex-Soviet research

Luxembourg. The European Communities' (EC) plan to spend ECU20 million this year on cooperation with scientists from the independent states of the former Soviet Union is gathering momentum. This makes the EC the second largest supporter of research in the new states after private financier and philanthropist George Soros.

The idea for the fund, first canvassed by CERN director Carlo Rubbia in October 1991, has taken nearly two years to bear fruit. The concept was championed by German and French research ministers Heinz Riesenhuber and Hubert Curien, who secured political endorsement from the OECD (Organization for Economic Cooperation and Development) in March last year. The commission of the EC finally approved the formation of a formal association of states last December.

Since then there has been an urgent search for projects, which must be collaborations between east and west. Although European laboratories are not eligible for funds, 54 projects have now been funded (ECU4 million), around one-third of the applications so far received. The largest award to a single ex-Soviet institute supports a ECU290,000 project on genes that control metastatic behaviour of cancer cells, to be run between the Institute of Gene Biology in Moscow

and the Fibiger Institute in Copenhagen.

Early applications were assessed by the EC's CODEST scientific committee, but a new panel will be set up to assess projects on which the remaining cash may be spent.

The association is semi-independent of the EC, which is only one of its 14 members. The others are the twelve member states plus Austria. The EC hopes that other countries of the European Free Trade Association will join, to boost the funds available. Germany is the only EC member state so far to supplement the European Commission's funds (with DM200,000), but new French research minister François Fillon informally indicated that he would match that figure.

The association's support, conceived as a means of stemming the brain-drain from the independent states, has made a good start, but the apparently inexplicable delays in its establishment drew wry comments last week from the Russian research minister Boris Saltykov. “We thought that Soviet bureaucracy was supposed to be the largest in the world, but the EC has shown great strength in this department,” he said.

The association has built-in obsolescence: it will cease to exist at the end of 1994 unless a positive decision is made to continue.

Alison Abbott

Physicians not fazed by AZT guidelines

Washington. Physicians in the United States have been advised by an expert panel to confer thoroughly with patients and then to use their own discretion in prescribing antiretroviral drugs, such as AZT, to patients who are outwardly healthy but infected with HIV.

The new guidelines replace those issued in 1990, which recommended routine prescription of AZT in all cases where the count of the marker CD4 cell has fallen below 500 per mm³. The new guidelines were drawn up last week by an independent panel convened by the National Institute of Allergy and Infectious Diseases (NIAID) and chaired by Merle Sande, the chief of medical services at San Francisco General Hospital.

"Doctors and patients should work as a team to design a treatment strategy that is both clinically sound and appropriate for each individual patient's needs, priorities and circumstances in daily life", Sande says. The panel, whose findings are widely seen as a response to April's preliminary report of the Anglo-French Concorde trial (see *Nature* 362, 483; 1993), emphasized that "the choice to accept or decline antiretroviral therapy ultimately rests with the patient", and

NIAID statement said.

The panel still recommends antiretroviral drugs for patients who report with symptoms of AIDS, with AZT recommended as the first line of treatment. But for patients who have low CD4 counts but no actual symptoms, the panel leaves prescription of the drug to the discretion of the physician.

Physicians working with HIV patients in major US cities say that the guidelines largely reflect their current practice of not prescribing AZT to patients on the basis of low CD4 counts alone. "I've always been somewhat cautious about prescribing these drugs," says Dr Donald Abrams, assistant director of AIDS practice at the San Francisco General Hospital. "The guidelines won't change my practice at all."

But Abrams criticises the panel for failing to say anything about the need for more clinical trials to establish better treatment options. His concerns are echoed by Dr Joseph Sonnabend, an AIDS physician practising in New York, who says: "The situation we are in now is a mess." Sonnabend says that many members of the panel were involved in earlier guidelines since proven to be premature. "A little self-criticism would

not have been out of place", he says.

Spencer Cox of the New York-based Treatment Action Group (TAG) says that the main impact of the guidelines will be in small-town America, and in inner-city clinics where patients may get little personal attention.

In a separate development, a National Commission on AIDS set up by statute four years ago issued its final report, attacking "governors, mayors, members of Congress, corporate executives, community or religious leaders" and "previous presidents" for failing to take a lead in tackling the AIDS epidemic.

Colin Macilwain

■ President Clinton has named Kristine Gebbie, a health care administrator in Washington state and former nurse, as his AIDS policy coordinator. AIDS researchers and activists welcomed Gebbie's reputation as a conciliator, but doubted if she would carry the clout to tackle powerful vested interests.

FDA joins market

Washington. The US Food and Drug Administration (FDA) is putting flesh on the bones of a programme that will, it hopes, allow it to raise over \$300 million in the next five years by charging drug companies for certain services it provides.

FDA's authority is the Prescription Drug User Fee Act, enacted last October (see *Nature* 359, 567; 1992). The money raised in user fees will boost current budgets and allow FDA to employ extra staff to speed the review of new drug applications.

FDA, which is about to begin invoicing companies, estimates that about 150 companies and 2,000 products will be caught in its net, but 70 per cent of the revenue is expected to come from 20 of the biggest US pharmaceutical companies. The agency expects to raise \$36 million in the first year of the programme, rising to \$84 million in 1997 with annual increases in the fees charged.

The money will be used to employ 620 extra staff — 280 for the Center for Drug Evaluation and Research, 300 for the Center for Biologics Evaluation and Research and the remaining 40 for the Office of Regulatory Affairs. An additional 80 staff will be required to handle the invoicing of companies and the collection of fees.

The overall objective is to meet performance goals defined in the statute, which industry may monitor more zealously than the Congress. FDA's interim goal is to deal with 55 per cent of new drug applications and product licence applications within twelve months in 1994, and to increase that proportion to 90 per cent in 1997.

Diane Gershon

Hopes for pan-European network

London. European research may soon have a continent-wide system of high-speed computer links as robust as its counterpart in the United States; those responsible for the national academic computer networks in 12 European countries are launching a new company to do just that.

Known as Delivery of Advanced Network Technology for Europe Ltd (or DANTE), the company is being set up by the members of the Réseaux Associés pour la Recherche Européenne (or RARE), a forum of national networking organizations based in Amsterdam. It will operate from Cambridge, England, with the goal of providing a comprehensive pan-European network for the academic, government and commercial research community as well as developing high-speed links to the United States. It will be accessed through and paid for by each country's own national academic network service.

DANTE will take over responsibility for various existing European networking services such as EuropaNET, a recently established 2 megabit per second network funded by the European Commission. The new company is also putting together a bid to the commission for a contract to establish a 34 m.b.p.s. network (enough capacity for video-conferencing) which would bring Europe-wide communication closer to the speeds available in the United States.

Several individual members of RARE

are already developing their own high-speed networks. In Britain, for example, work is currently under way on a 140 m.b.p.s. network SuperJANET, funded by a £5 million grant from the government through the Higher Education Funding Councils.

Many of the individual networks have already developed bilateral links, both with counterparts in Europe and with the various research networks in the United States. But progress at the European level has been disappointing, partly because of the complexities of dealing with highly political telecommunications agencies and partly because of the high costs of long-distance data-links, which can be five or six times more expensive than in the United States.

DANTE is seen as an attempt to finesse these problems. It hopes to act as a central source of technical advice, to use contacts built up through member organizations to address political issues and to provide a single applicant for EC funds, as well as to negotiate with US and other networks.

Supporters of DANTE hope that it may also attract funding by offering the academic community as a guinea-pig for more ambitious plans to develop a pan-European system of "information highways", an idea parallel to that being pushed by vice-president Albert Gore in the United States, which was proposed two weeks ago by the president of the Commission, Jacques Delors.

David Dickson

Supreme Court ruling receives warm welcome

Washington. The US Supreme Court has clarified the rules on the use of scientific evidence, making judges responsible for ensuring that "any and all scientific testimony or evidence admitted is not only relevant, but reliable."

The ruling is expected to improve the quality of scientific evidence heard throughout the federal judiciary, where unsound science and competing scientific claims have presented persistent problems.

Last week's ruling arises from the Supreme Court's adjudication on the admissibility of evidence in a damage suit brought against Merrell Dow Pharmaceuticals on behalf of two San Diego children born with shortened or missing limbs to women who took the now-discontinued drug Bendectin to ward off morning-sickness during pregnancy. The case began in 1984.

During the trial of *Daubert v. Merrell*,

lawyers for the families wished to present evidence from pathologists and epidemiologists that would have supported the plaintiffs' case, but were denied by two Californian courts on the grounds that the evidence had not been peer-reviewed, published or generally accepted in the scientific community.

That is the essence of the 70-year-old legal standard called the "Frye test", which allows as evidence only that based on scientific methods recognized as reliable. Rejecting that standard, the Supreme Court says the test should be whether the methods used to reach conclusions are sound.

That means that US judges must now sift reliable from ill-founded science by considering whether the claim is testable, whether it has been empirically tested and whether the testing has been carried out according to a scientific methodology.

"The ruling is wonderfully simple. If a theory fails to meet any of these three questions, it doesn't qualify as science and, therefore, doesn't make it into the courtroom," said Steven Gallagher of the Carnegie Commission on Science, Technology, and Government, which recently published a set of guidelines to help judges distinguish between good and bad science.

Including the Carnegie Commission, no fewer than 22 organizations and individuals offered the court a range of opinions on what kind of evidence should be allowed in courts. Among opinions supporting Merrell Dow were those of the American Medical Association, the National Academy of Sciences and the American Association for the Advancement of Science, all of which applaud last week's ruling.

The decision follows a period during which US courts have been criticized for reaching decisions at odds with scientific reality. The controversy peaked last year in the case of a claim that a spermicide had caused birth defects. The court had held that "sufficient evidence of causation", and not science, should influence the law.

Bert Black, who chairs the American Bar Association's Standing Committee on Scientific Evidence, believes the ruling will create "a real binding distinction between quack experts with crackpot theories and true scientists with sound reliable evidence."

Last week's decision seemed to satisfy both sides. The plaintiffs will not be barred from presenting their evidence, but that will be heard only if the courts agree that the science is sound.

Others are pleased by the ruling. US businesses, which have complained that the threat of unreliable testimony by "hired guns" has led to unwarranted jury awards in personal injury and malpractice lawsuits, are among them. But last week's ruling is also claimed as a victory by the groups of lawyers, science historians and epidemiologists who supported the plaintiffs.

Quick to point out that Galileo was persecuted for advocating heliocentrism, they say the decision will force courts to be more flexible with scientific mavericks whose work has not been accepted by peer-reviewed journals. Given that much of the information relevant to legal cases is new to science, the ruling will also make it easier for litigants to put before juries evidence based on novel or experimental techniques. The new development will also affect the use made by courts of evidence from DNA fingerprinting, about which there has been controversy in recent years.

The ruling technically applies only to federal courts, but is expected to be followed by state courts as well. The practical difficulties have yet to emerge. Certainly it will make more work for the already-overburdened courts. Experts say judges will have to schedule even more preliminary hearings and hire experts to assist them in deciding what evidence to allow.

Susan Greene

Europe's research plan still up in air

Luxembourg. The European Community (EC) multi-billion dollar fourth framework programme for research (1994–98) seems to be heading for the delays that plagued its predecessor. The twelve research ministers meeting in Luxembourg last week failed to agree on what the total budget should be and how it should be divided.

The European Commission's own plan recommended a ECU13.1 billion budget, of which most (83.4 per cent) would be allocated to a research and development programme broken down into seven categories: information and communication technology (29.8 per cent); industrial technology (13.7 per cent); transport research (2.1 per cent); life sciences (10.1 per cent); environment (7.4 per cent); energy (19.3 per cent); and socioeconomics (1.0 per cent).

The commission would have divided the rest of the money between the three other activities of the framework programme: international cooperation, including that with eastern Europe (6 per cent); dissemination and optimization of results (4.6 per cent) and human capital and mobility (6 per cent).

No government demands major shifts in this allocation, but small demands can force stasis when they are in contradictory directions. Denmark, which held the rotating EC presidency until 1 July, put forward a compromise designed to bridge the various differences, but it did not succeed.

Thus Britain, France, Germany and the Netherlands, all of which have strong telecommunications industries which stand to benefit from future contracts, held out for

more spending on information technology. Germany alone stood out for a much higher allocation to environmental research (12.3 per cent, compared with the Danish compromise of 8–10 per cent). Britain and France want lower spending on the life sciences, Belgium more on energy.

Many countries also want to see a reduced share for international cooperation and dissemination of results. Most countries are not yet ready to commit themselves on the total sum to be allocated to research from the what the EC call their internal policy budget, which also covers training and networking. The commission proposes that research should take about 60 per cent of this (ECU13.1 billion), but many want to reserve judgement until the EC's general budget as a whole is clearer.

Optimists still hope that disagreements can be ironed out in time, for approval at the next council of ministers meeting in December. Pessimists remember the political delays that held up approval of the third framework (1990–94). The impending ratification of the Maastricht Treaty may cause further delay. The treaty requires 'codecision', or the agreement on all significant issues of all three EC governing bodies — commission, parliament and council of ministers.

Codecision, while democratic, will probably lengthen all EC procedures. And elections due next June are likely to disrupt the smooth functioning of the Parliament, which could delay approval further if the framework programme falls seriously behind schedule.

Alison Abbott

More red tape than ever for Russians travelling abroad

Moscow. Crossing the borders of Russia has become almost as great a problem for Russian scientists as their financial troubles. The result is that most people agree that the bureaucratic obstacles to travelling abroad now compare with those familiar during the Communist regime.

Thus Ia Vaiskova, the director of the Travel-Grant Program of the International Science Foundation (established by the philanthropist George Soros), says that in the few months of the programme's operation, a hundred scientists were able to attend conferences abroad, but that roughly the same number of trips had to be cancelled because travel documents could not be processed in time.

The difficulties have been mounting since the turn of the year, when the Russian government set out to exchange existing USSR foreign passports (necessary for travel abroad) for Russian documents. The need for this exchange is still not clear: the new passport is the same as the USSR passport, but with a different serial number.

Officials claim that the exchange is needed to give up the old exit-visa system: all new passports are cleared by the successors of the KGB, and do not require a stamp on leaving Russia. But the whole business has turned into a bureaucratic nightmare and an opportunity for bribery on a scale unprecedented even in Russia.

As a result, Russian scientists cannot attend many conferences and other events abroad. The International Science Foundation now makes it a rule that travel-grant recipients must have a foreign passport ten days before the departure date, to allow the paperwork to be processed. Otherwise, a scientist may lose a travel grant.

The situation is especially devastating in Moscow. Astrophysicist Alexei Fridman had to abandon plans to attend a conference to which he had been invited as a speaker because the passport exchange procedure requires at least two months. But his colleagues at St Petersburg were able to go, because travellers there can use the old passports provided they are from the most recent issue, known as Series 21.

Some Muscovites accordingly seek to leave Russia from other states of the former Soviet Union, such as Belarus. Others get out by bribery. An exit-visa stamp in the old, series-21, passport costs US\$80–\$160, the "rush fee" for a new passport ranges from \$500 upwards. Even worse, these services are by no means reliable. Every day, 60 to 80 people with forged stamps and passports are turned back from Moscow International Airport.

The difficulty in obtaining passports is

not the only one. Fearful of being flooded by Russians now that exit-visas have been abandoned, the consulates of many countries have tightened their visa requirements. It is almost impossible to visit some countries at short notice. While the US consulate is relatively cooperative and there are few problems with travel to Israel, many European and Asian countries have erected barriers that are almost impossible to overcome.

Vaiskova says that restrictions are especially onerous with China and Japan. Their consulates refuse to start processing documents until they receive approval from their ministries of foreign affairs, which usually takes a month. Among European governments, the International Science Foundation encounters most problems with the French and Italian consulates.

It is difficult to find a word other than "harassment" for the treatment of Russian citizens at the French consulate. Sometimes, according to Vaiskova, scientists are invited

to pick up their visas an hour after the departure time indicated on their airline tickets. But many Russian scientists also complain about the humiliation they must suffer at the hands of the German staff.

Earlier this year, the Swiss consulate was highly regarded, but now it implements a quota system, awarding only 20 visas a day to the citizens of a country of 150 million people. As a result, a person needs to queue for several weeks to be allowed to deliver papers to the consulate.

Most consulates say they do not distinguish between the purposes for which Russians wish to travel, and that they treat immigrants, businessmen, students, scientists and tourists equally. Equality would be welcome if everybody was treated equally well. But if everybody is equally harassed, that is an egalitarianism all too familiar to Russians.

Michael Monastyrsky from the Institute of Theoretical and Experimental Physics, who is now waiting for a visa without being sure that he will receive it, even believes that the inconvenient and even brutal rules of the Communist past were better than the present bureaucratic abuse. "At least then you knew what could be done and what could be expected", he says.

Vladimir Pokrovsky

Bomb attacks on US researchers

San Francisco. US federal agents and university officials have warned researchers to be alert for suspicious packages after two prominent scientists were seriously injured by separate mail-bomb explosions in the same week.

On 22 June, a 8 1/2 inch by 11 inch padded manila envelope exploded in the hands of 59-year-old Charles Epstein, a geneticist at the University of California, San Francisco, as he opened the package at his home. Two days later, a similar bomb critically injured David Gelernter (38), an associate professor at Yale, as he was opening mail in his office at the campus computer sciences building. Epstein lost three fingers and suffered a broken arm and abdominal injuries. Gelernter was badly injured in his chest and abdomen and may have a permanently damaged eye and hand.

The Federal Bureau of Investigation (FBI) links the two bombings to each other and to a dozen unsolved attacks between 1978 and 1987. Special agent Tim Bezick in San Francisco says the bombs' materials, construction, packaging and targets all point to the same individual or group. An earlier federal investigation was disbanded for lack of leads.

The FBI says it has found no link between the two researchers recently attacked, nor any motive for the bombings. The earlier explosions had targeted seven uni-

versities, a computer store, Boeing Corporation and United Airlines. Bezick says the FBI has no idea why two attacks should suddenly have followed six years of quiet except the conjecture that the person responsible had been in prison or some other institution.

Bezick said it was not clear why the bomber had targeted a geneticist (Epstein) for the first time. A telephone threat the afternoon following Gelernter's injury seemed aimed at his brother Joel, who is a geneticist at Yale, but investigators believe that call may have been from a crank.

The FBI has sent an e-mail warning to university administrations urging them to warn staff about strange packages in the mail. Officials at Massachusetts Institute of Technology, Harvard University, the University of California at Berkeley, Yale University and the University of California at San Francisco all said they are taking special security precautions, but they also said they hope to avoid panic.

Federal agents warn scientists to watch for rigid packages with markings such as "personal" or "confidential" a hand-written or poorly typed address, excessive postage, a lopsided shape or excessive securing material (such as string or tape). They warned researchers not to touch or cover such a package, and to move people away from it rather than to move the package itself.

Sally Lehman

Duesberg: rights and wrongs

SIR — I have no quarrel with the policy that gives an editor freedom to publish or to reject whatever material may be submitted to him, but for him to reject an intended publication and then to subject its author to the kind of criticism levelled at Dr Peter Duesberg in your recent leading article (*Nature* 363, 109; 1993) seems to me at best unnecessary and at worst grossly unfair.

I am no friend of Duesberg, or of his campaign on the causation of AIDS, but your treatment smacks of the “non-religious Inquisitions of the twentieth century” referred to by Psimopoulos and Theocharis, ironically in the same issue of *Nature* (363, 108; 1993), in their comments on the Vatican’s tardy rehabilitation of Galileo.

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SIR — You state that Peter Duesberg wrongly uses tendentious arguments to confuse understanding of AIDS, that his debating technique is intolerable because he asks unanswerable rhetorical questions and that he should stop. The tone and logic of your leading article seem inappropriate to a scientific journal, and *Nature*’s position on Duesberg’s theory seems prejudiced.

The rhetorical questions Duesberg has posed are in fact statements — that the widely accepted hypothesis that HIV is the cause of AIDS is not consistent with the facts. If, for instance, someone asks the question “Why cannot the theory explain the facts A, B, C . . . ?”, that is not only a stimulant of further research, but a serious objection against the theory.

The reasoning behind Duesberg’s theory occupies 60 pages of text and refers to more than 600 papers by other authors (*Pharmac. Ther.* 255, 201–277; 1992). The attempt to prove that the theory is wrong, based on references to a few studies by Duesberg’s opponents, is not persuasive: there are no criteria to estimate these as more authentic than those to which Duesberg refers.

You state that Duesberg’s work has not been published because it conflicts with *Nature*’s obligation to provide readers with authentic information. Duesberg adduces data from works of other authors and official sources of statistical information to support his theory. In effect, his work expresses original interpretations of well-known and published facts, and the statement that the work does not meet the criterion of authenticity is at variance with logic.

It is clear from the context that the decision to reject the work is based on the

conclusions of referees. It is quite easy for an editor to predict the negative response of a referee to an unorthodox work, and the choice of referee(s) is almost equivalent to a final decision to reject the work.

The real motives for the rejection of Duesberg’s paper remain unclear. Neither the peculiarities of his rhetorical style nor “non-authenticity of information” can explain why the paper has been rejected. The real cause seems to be connected with the question (non-rhetorical and answerable) of whose interests may suffer if Duesberg’s theory is true.

Duesberg’s paper highlights a much more general problem: the effectiveness of the procedures used by scientific journals to select papers for publication. Even for ordinary works, the modern system seems ineffective (see, for example Ernst *et al.* *Nature* 363, 296; 1993). If a work expresses an unorthodox view, its chance of being published is quite small. As the history of science demonstrates, most landmark works have been at first regarded as wrong and intolerable. Open discussion of this problem in *Nature*’s pages would be very useful.

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SIR — We congratulate you on the leading article in *News and Views* on Duesberg’s right of reply. We have both been acquainted with Duesberg since the early 1970s, and agree that his high-calibre research papers published before he became a campaigner should not be forgotten. However, in this respect, the general reader should keep in mind that, in addition to his campaign against HIV, Duesberg’s views on the existence of oncogenes also conflict with what is generally accepted as the most reasonable and scientifically sound explanation of cellular carcinogenesis.

Given his early successes, one can only speculate sadly on the excellent contributions Duesberg might have made in the past decade had he chosen not to follow the path of righteousness (or wrongness, as the case may be). On HIV and AIDS, Duesberg has certainly raised cogent questions about the pathogenesis of the disease, as have many other scientists who have followed the more accepted path: first the question, then the experiments and then the conclusions. A recent report in *Nature* by Pantaleo *et al.* (362, 355–359; 1993) provides an excellent example of how a great deal of arduous work contributes to solving the mystery of locating HIV when we can’t detect it in the blood. In the past decade, Duesberg has published very little material to back up his claims. Recently, he has regrettably

moved one step beyond campaigning, by making false accusations against scientists who do not happen to share his beliefs. These colleagues have adequately answered back (see *The Lancet* 341, 1222–1224; 1993). A. G. Dalgleish’s last paragraph is rather strongly worded, but it spells out the present unfortunate situation.

Your subtitle ends: “He should stop”. Or, we submit, “should he be stopped?” For example, should he somehow be prevented from appearing on television to misinform individuals who are at risk from the disease? One approach would be to refuse television confrontations with Duesberg, as Tony Fauci and one of us managed to do at the opening day of the VIIIth International Conference on AIDS in Florence. One can’t spread misinformation without an audience.

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Eppur si muove

SIR — Psimopoulos and Theocharis¹ (*Nature* 363, 108; 1993) point to possible error in Galileo’s contention of the “triumph absolutely” of the Copernican theory. They cite Einstein’s coordinate relativity to question whether one can say the Earth moves about the Sun or vice versa. But the essence of the Copernican model is that the Earth and the other planets revolve around the Sun, rather than older tradition that the Sun and the other planets revolve around the Earth. The two models differ in this essential topology, and cannot be converted by any reasonable geometric translation. Galileo’s essential contention was the incorrectness of the theological model: an immovable Earth at the centre of the Universe, with the Sun and planets in their orbits about it.

Regardless of the relativity of coordinate systems, in the Copernican sense, *eppur si muove*.

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Academic equality

SIR — “If top universities truly want to be seen to lead the field in sex equality . . .”: so *Nature* on 27 May (363, 288; 1993). But surely what is needed is equality of opportunity. One may hope that this will lead to equality of achievement, but possibly it may not, and it cannot be taken for granted that the two must be the same.

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New genetics means no new ethics

The opinion that genome sequencing will create novel ethical problems is mistaken; the techniques are hardly novel, and are unlikely to be more troublesome than those of the genetic manipulation of bacteria twenty years ago.

THESE days, everybody seems to have an opinion on the ethics of genetics. And most opinions are portentous, laden with apprehension and downright distrust. The new genetics seems to be as widely feared as were the development and deployment of nuclear weapons in the 1950s. It may not be long before geneticists enjoy the popular reputation that then attached to Stanley Kubrick's celluloid character Dr Strangelove, and when biotechnology enterprises that have proudly chosen corporate names including 'GENE' or 'GEN' will be scampering for politically more correct alternatives. But the widespread fear of genetics cannot be justified. On the contrary, the research community should speak out strongly to defend the good sense of what it is about.

Present difficulties go back nearly two decades to the Asilomar conference at which, in 1975, a group of molecular biologists recommended a moratorium on genetic manipulation while arrangements were made to regulate recombinant-DNA techniques. Even with the largely reassuring knowledge that experience has brought, that was a wise decision. There *might* have been hidden dangers in the innocent manipulation of microorganisms. Moreover, the malevolent manipulation of microorganisms could still make possible the manufacture of dangerous biological agents. That is the case for continuing to regulate genetic manipulation.

Asilomar has nevertheless left an awkward legacy. Without precedent, the research community invited legislation for and regulation of its own activities. Valuable though the outcome has been in making genetic manipulation widely understood and almost as widely acceptable, the precedent is now taken to apply to all novel research in biology. But experience has also shown that governments find it easier to create regulations than to dispense with them when the hypothetical dangers they are designed to avoid are shown to be negligible. Asilomar has become a lightning-rod for generalized anxiety about genetics in any form.

Even so, it is not easy to see why the more recent development of techniques for determining the nucleotide sequences of genes, followed by the ambition to sequence the whole human genome, should be of such interest to the people now called 'ethicists'. Part of the trouble is the difficulty of explaining the difference between discovery and its application; there is a temptation to believe that the first sequence of a human genome will be quickly followed by the widespread application of that knowledge

without material impediment, such as cost.

There is also a temptation to believe that new laboratory techniques mean new applications and thus new ethical problems. But that is not the case. Indeed, the availability of gene sequences, and ultimately of the sequence of the whole genome, will not create ethical problems that are intrinsically novel, but will simply make it easier, cheaper and more certain to pursue well-established objectives in the breeding of plants, animals and even people. That is the principle by which the research community should stand.

The attention paid in the past few years to the nucleotide sequences of genes involved in the inheritance of genetic diseases is a good example. The molecular causes of conditions such as sickle-cell anaemia, the various thalassaemias, Huntington's disease, fragile-X syndrome and cystic fibrosis have all been determined in the past few years. But this new knowledge has not created novel ethical problems, only ethical simplifications.

Long before gene sequencing, it had been recognized that these conditions were genetic: they run in families. It became standard practice to warn putative carriers of disease genes of the risks of conceiving children. Is it not a welcome improvement of that practice that genetic counsellors no longer have to rely on inference, based on imperfect family histories, in giving their advice? Or that when the impending birth of a child with a genetic disease can be determined by amniocentesis, that most governments should allow abortion?

But may not such practices be carried too far? One fear is that the avoidance of births of individuals carrying genes supposed 'defective', but especially the elimination of those genes from a population, may unknowingly rob society of valuable genetic endowments. The heterozygous advantage (resistance to malaria) of the sickle-cell gene is the classical example. The possibility that the 'gene' for schizophrenia, which must be commonplace if it exists, may confer advantages not yet recognized on those in whom the overt disease does not develop is often cited as a more serious pitfall.

The truth is not simply the practical consideration that it will be a long time before the genetics of these psychiatric conditions is understood, but that geneticists themselves are likely to be the first to recognize the dangers of interfering with the natural flow of genes within a population before the social implications are understood. Indeed, only geneticists can recog-

nize the dangers. More general recognition of them should help enormously to modify for the better the now too-common view that genes spell genetic trouble whenever they appear in a non-standard form. (On the other hand, to refrain from calling the Huntington's gene 'abnormal' is to concede too much to political correctness.)

There are more subtle dangers. Because the nucleotide sequence of a person's genome makes it possible to discriminate between people, genetic sequencing could become the basis for refusing individuals medical insurance or even employment in some occupations. All that is true in principle, but there are three good reasons why the truth does not amount to a novel ethical challenge.

First, insurers already discriminate against people who smoke cigarettes, or who are infected with HIV; why should they not also discriminate against, say, people with the particular structure of the LDL receptor known to be responsible for early-onset familial heart disease? Second, would it not be beneficial that people carrying the heart disease gene should not be employed on heavy manual work (or better, that they should be steered towards drug treatment)? Third, there are practical problems: when 100,000 genes have been sequenced, how many will insurers or employers include in their genetic screens, and at what cost?

Then there is the matter of eugenics. Genetic screening by amniocentesis does not decrease the frequency of an unwanted recessive gene in a population, but may even increase it. And of necessity, gene therapy designed to improve on the defective function of some class of somatic cells can have no effect on the condition of others than the individual patient. But what if it were possible (as it is not, yet) to modify germ cells in the human reproductive system?

Reference to Hitler is common at this stage, but is mistaken. What serious geneticist would at this stage advocate the artificial selection of a particular genome throwing away the benefits of hybrid vigour and genetic diversity? But would it not be of great value if the frequency of the haemophilia gene in the human population could somehow be reduced? Even if (when a safe way of doing that has been found) by germ-cell manipulation? Geneticists are fond of saying they will "never touch the germline". That is unwise.

John Maddox

Summary of a talk at the European Federation of Biochemical Societies, Stockholm, 5 July.

Current problems at high T_c

D. K. Christen and J. R. Thompson

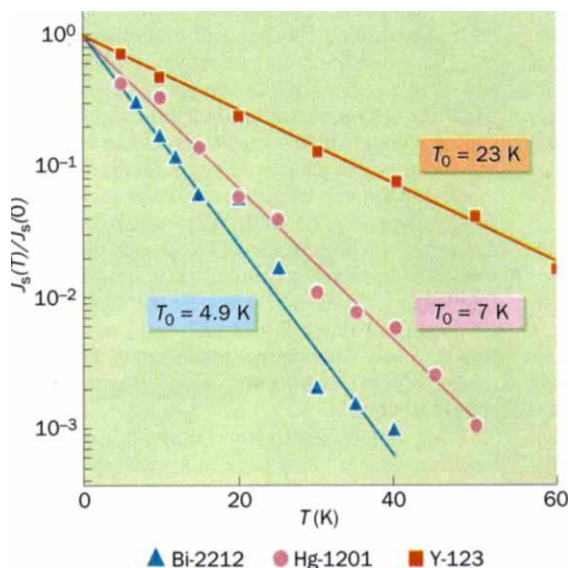
IN March this year a new family of superconducting materials, the mercury-based layered copper oxides, came to light¹; the latest of the brood has set a new record of 133 K (−140 °C) for the superconducting transition temperature². As befits Mercury, also the winged messenger of the gods, the response of the superconductivity community has been swift indeed. The present fervour harkens back to the heady days of late 1986 and early 1987 when work on high-temperature superconductors virtually exploded. On page 129 of this issue, Umezawa and colleagues³ provide some of the information that is so urgently needed by characterizing the current-carrying capacity of one of these superconductors.

What do we know at present about these new superconductors? Structurally they contain $n = 1, 2$ or 3 adjacent copper oxide sheets, forming a compositional series $\text{Hg}_1\text{Ba}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+2+\delta}$. As in the similar thallium- and bismuth-based materials, the superconductivity undoubtedly originates in the electronically (hole) doped copper oxide layers. And like the earlier families, the mercury-based counterparts have a superconductive transition temperature T_c that increases with layer number n , with values of 95 K, ~115 K (ref. 4) and the record high 133 K for $n = 1, 2$ and 3, respectively. These high values, especially for the $n = 1$ endmember, known as Hg-1201, which can be readily and reproducibly synthesized, account in part for the current excitement in this field.

Another potential attraction is their relatively simple tetragonal structure⁵, which lacks the complexity of Cu–O 'chains' in Y-123 ($\text{Y}_1\text{Ba}_2\text{Cu}_3\text{O}_{7-\delta}$) or superstructural modulations as in Bi–Sr–Ca–Cu–O. Moreover, the relatively close spacing between sets of Cu–O layers gives hope that the sets of planes are electrically well coupled together. Such a happy circumstance should make the superconductor more isotropic and improve its properties relative to the highly anisotropic thallium- and bismuth-based materials. Umezawa and colleagues show that, as hoped, the magnetic irreversibility line in Hg-1201 is higher than for these. The irreversibility line marks the boundary in the plane of magnetic field H and temperature T where the areal density of loss-free current J tends towards zero; this line should be as high as possible to permit applications at high fields and readily

achievable temperatures (ideally, in the liquid nitrogen range, but we are a long way off as yet).

So much for the good news. The Hg-based materials, or at least the Hg-1201 member, also possess some sobering aspects showing that applications are unlikely to be easy. First, supercurrent conduction between randomly oriented grains is poor, as demonstrated by Umezawa *et al.*³ and independently by others⁶. This 'weak-linkage' between grains with



The capability to conduct electric current deteriorates rapidly with temperature T . The persistent supercurrent density $J_s(T)$, relative to its value at absolute zero, decreases roughly exponentially as $\exp(-T/T_0)$, because of flux creep. With a magnetic field of 1 tesla, intragrain results for polycrystalline Hg-1201 are compared with data for single crystals of Y-123 and Bi-2212, with the magnetic field perpendicular to the Cu–O planes (after ref. 6).

random orientations seems ubiquitous in the high- T_c superconductors that have very short superconducting coherence lengths. Second, the persistent current density flowing within individual grains decreases rapidly with field and temperature, falling off nearly exponentially as $\sim \exp(-T/T_0)$ (see figure).

As is evident, the Hg-1201 material behaves much like certain well studied, as-grown superconductors and lies in the middle of their range. The likely cause is weak pinning of magnetic flux lines (vortices). The resulting 'giant flux creep'⁷, which has been observed experimentally for Hg-1201, strongly limits the attainable current density. Unless overcome by artificially induced pinning sites for vortices, this snag will impose an ultimate limit on possible applications. One encouraging finding is that defects generated by neutron irradiation have produced an order-

of-magnitude increase in the intragrain current density in Hg-1201 materials⁸. Overall, Hg-1201 shares several features with other high- T_c materials that make their application challenging, and it is likely that this also applies to the other members of the mercury family.

What directions are appropriate now and what needs to be done? These new superconductors have both intrinsic scientific interest and overlapping technological potential. The fundamental, anisotropic properties of the materials should be established through study of single crystals, aligned arrays and epitaxial films. These features include the fundamental superconducting parameters: the coherence lengths, magnetic penetration depths, thermodynamic critical field and so on; these will also quantify the degree of anisotropy. A next step is to relate these parameters to optimized, ideal defects for pinning vortices and maximizing the intragrain (rather than intergrain) current density.

Methods for materials modification and defect generation include irradiation with particles (energetic ions, neutrons and so on) and novel processing techniques (such as formation of very small second-phase particles or strain-induced crystalline faults). These types of basic systematic studies should define the intrinsic limits of current carrying capability in the materials.

The problem of 'weak links' severely impedes technological applications, so methods must be devised to synthesize highly textured (crystallographically aligned) materials, in order that the planar supercurrents in one grain can bridge the boundary and flow into adjoining grains. Therefore, we must define the thermophysical properties as they relate to the formation of textured, continuous conductors; in Hg-1201, the block-like microstructure is likely to make mechanical texturing methods difficult. The chemical and mechanical stability of the materials under practical operating conditions needs to be established as well. Finally, reliable and reproducible synthesis routes must be developed: forming the Hg-1201 endmember seems to be straightforward, but formation of the $n = 2$ and 3 phases has so far proved to be a much more difficult task. ►

1. Putlin, S. N., Antipov, E. V., Chmaissem, O. & Marezio, M. *Nature* **362**, 226–228 (1993).
2. Schilling, A., Cantoni, M., Guo, J. D. & Ott, H. R. *Nature* **363**, 56–58 (1993).
3. Umezawa, A. *et al.* *Nature* **364**, 129–131 (1993).
4. Meng, R. L. *et al.* *Physica C* (submitted).
5. Wagner, J. L. *et al.* *Physica C* (in the press).
6. Paranthaman, M. *et al.* *Physica C* (in the press).
7. Yeshurun, Y. & Malozemoff, A. P. *Phys. Rev. Lett.* **60**, 2202–2205 (1988).
8. Schwartz, J. *et al.* *Phys. Rev. B* (submitted).

Mother Nature has intrigued us yet again with new superconductors having remarkable properties and still higher transition temperatures. As with their predecessors, these mercury-based copper oxides have much promise but they also have many problems. Perhaps problem and promise are just different perspectives on the same thing — but the real

challenge that now remains is to translate the first into the second. □

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ASTROPHYSICS

Early stages of star formation

Stephen E. Strom

ASTRONOMERS have long known that stars are born in dense, cool clouds of molecular hydrogen, but until now they had not observed the direct precursors of newly formed stars: condensations of gas and dust within molecular clouds known as protostellar cores. Two groups now claim to have found protostellar cores in the initial stages of collapse — Chini and colleagues¹ have located a 50-solar-mass core in Orion (about 460 parsecs from us) and Andre and colleagues² have found a 1-solar-mass core in the nearby Ophiuchus star-forming complex. Both groups made their discoveries with the Institut de Radio Astronomie Millimétrique and the James Clerk Maxwell telescopes. Observations at millimetre and submillimetre wavelengths enable them to estimate the temperature, density and size of the putative protostellar cores from the emission arising from cold, micrometre-size dust grains and molecular gas. The critical point is the detection of cores both cold and dense enough to collapse against their own internal pressure.

Although it has been recognized for more than 40 years that stars form within opaque complexes consisting of gas (primarily molecular hydrogen) and dust grains a micrometre or less in diameter, it is not clear exactly how these very diffuse entities (of size 10^{14} to 10^{15} kilometres, and densities of only 100 to 1,000 molecules per cubic centimetre) give birth to large numbers of new stars with radii 100 million times smaller. Locating structures — protostellar cores — that are intermediate in size and density between molecular clouds and stars, and cataloguing their physical characteristics (mass, radius, rotation and internal motions) is seen as a key to progress.

Work by Myers and collaborators³ implicates 'molecular cores' (of size 0.3×10^{13} kilometres and densities 10^4 to 10^5 molecules per cubic centimetre) embedded within larger molecular clouds as the direct precursors of individual stars. Shu and co-workers⁴ calculate that cores similar to those observed by Myers can

arise naturally as gravitational and magnetic forces act over times of 1 to 10 million years on the lightly ionized molecular material of the clouds. In their picture, Myers cores represent electrically neutral pockets of molecular gas that have shed their magnetic fields, thus making them more susceptible to gravitational collapse owing to the loss of internal magnetic pressure.



The Trifid Nebula in Sagittarius — site of recent star birth.

But recent observations suggest that, at least in some cases, stars may form from clumps much smaller than a Myers core. In particular, infrared surveys^{5,6} capable of penetrating the opaque dust pervading molecular clouds have revealed dense newly formed stellar clusters, still embedded within their natal molecular clouds. In some clusters, the average stellar density exceeds 10 stars per Myers core, and in some cluster sub-regions the local stellar density is even higher. It seems likely that dense groups of stars originate where there are large numbers of small, dense protostellar cores, perhaps more similar in size to the cold condensations described by Chini *et al.* and Andre *et al.* than to the larger Myers cores.

The presence of small, dense condensations a tenth the size of a Myers core has been suspected for some time. For example, Falgarone and collaborators⁷ have made a number of millimetre-wave

observations at high angular resolution in nearby star-forming molecular clouds. From observations of carbon monoxide emission, they argue that the clouds are highly clumped, with clear evidence for distinct structures as small as 10^{11} km across. Most of these clumps seem to be transient structures, short-lived density enhancements analogous to waves on the ocean surface. A few, however, may be dense enough and cold enough to enable gravity to overcome internal pressure forces, and for stellar collapse to begin. The newly identified condensations may well be our first concrete examples of this.

Once collapse has begun, astronomers believe that the core quickly develops a dense central concentration made up of the low-angular-momentum material in the core. The central 'protostar' is soon surrounded by a circumstellar disk of core material with higher angular momentum. Over time (10,000 years for the most massive stars; 1 million years for stars like the Sun), the protostellar mass increases both by accretion through the disk and by infall — directly onto the star, and onto

the disk⁴. The onset of disk accretion initiates an energetic mass outflow in the form of a highly collimated wind emanating from the inner regions of the disk and emerging along the disk poles. The wind is powerful enough to remove a good fraction of the remaining material contained in the protostellar core, and may in fact fix the final mass of the star by removing the reservoir of available protostellar material.

Working out the link between these cores and resultant stars and stellar groups is the current focus of attempts to understand how stars form. Does the spectrum of stellar masses in a star-forming region reflect simply the distribution of protostellar core masses, or something more subtle, such as a range in core initial conditions? Do interactions between protostellar cores (for example, core mergers analogous to the galaxy mergers thought to produce massive elliptical systems⁸) alter the shape of the stellar mass spectrum in regions of high protostellar core density? If so, does this account for the tendency for high-mass stars to be found in regions of high stellar density? Can the process that produces small, dense protostellar cores

1. Chini, R. *et al.* *Astr. Astrophys.* **272**, L5–L8 (1993).
2. Andre, P., Ward-Thompson, D. & Barsony, M. *Astrophys. J.* **406**, 122–141 (1993).
3. Myers, P. C. & Benson, P. J. *Astrophys. J.* **266**, 309–320 (1983).
4. Shu, F., Adams, F. C. & Lizano, S. *A. Rev. Astr. Astrophys.* **25**, 23–81 (1987).
5. Lada, E., Strom, K. M. & Myers, P. C. in *Protostars and Planets* (eds Levy, E. H. & Lunine, J.) 245–278 (Univ. Arizona Press, Tucson, 1993).
6. Strom, K. M., Strom, S. E. & Merrill, K. M. *Astrophys. J.* (in the press).
7. Falgarone, E., Phillips, T. G. & Walker, C. *Astrophys. J.* **378**, 186–201 (1991).
8. Larson, R. in *Fragmentation of Molecular Clouds and Star Formation* (eds Falgarone, E., Boulanger, F. & Duver, G.) 261–273 (Kluwer, Dordrecht).

RÉSUMÉ

Carbon credits

THE vast tracts of forest and peatland in the former Soviet Union have been largely left out of reckonings about the carbon cycle and global warming, say T. P. Kolchugina and T. S. Vinson (*Global biogeochem. Cycles* **7**, 291–304; 1993). Drawing upon Soviet databases and maps of the areas covered by forest, tundra, steppe and arable land, they conclude that the forests' net effect could be to mop up as much as 485 million tonnes of carbon per year, about half the amount that is released yearly by destruction of tropical forests. Kolchugina and Vinson point out that there could be a marketing opportunity here — industrial nations whose carbon emissions exceed internationally agreed limits could purchase carbon credits, rather like buying a mass to offset their sins, by paying for the preservation and careful management of the Russian forests.

Deceptive difference

Two years ago A. R. Zangerl and colleagues proposed an explanation as to why wild parsnip, *Pastinaca sativa*, should bother producing seedless fruits (this phenomenon is widespread and is known as parthenocarpy). It was that the parthenocarpic fruits, which contain lower concentrations of deterrent furanocoumarins, serve as decoys for viable fruits in the face of hungry insects. A. Traveset has revisited the problem with a different plant, *Pistacia terebinthus*, and comes up with a variation on the theme (*Evol. Ecol.* **7**, 357–361; 1993). She finds that chalcidoid wasps that lay their eggs in the fruits cannot tell the difference between the viable and the inviable ones, and that the higher the proportion of parthenocarpic fruits in a crop, the lower the proportion of wasp-damaged seeds. So it seems that deceit can have several consequences — some of them fruitful.

Bend sinister

E. V. CURTO *et al.* (*Biochem. Biophys. Res. Commun.* **193**, 688–693; 1993) have determined by NMR the structure in solution of a decapeptide hormone from the ovaries of mosquitoes. This substance, the trypsin-modulating oostatic factor, stops egg development by terminating the synthesis of a protease. The peptide contains seven prolines, and similar sequences found in various proteins contain a polyproline helix with β -turns. The decapeptide has no turns, however, probably because it is too short, and turns out to be a left-handed helical rod, 3 nm long. Based on the behaviour of proline-rich fragments found in bacteria, Curto and co-workers speculate that their hormone may function by binding to an SH3 domain in its receptor. High-resolution structures of SH3 domains now emerging might allow that conjecture to be explored.

account naturally for the observed high frequency of binary stars, or is another process responsible for binary and multiple star formation?

Identifying protostellar cores in different star-forming environments and characterizing their physical properties will tie up a lot of observing time with millimetre- and submillimetre-wave instruments capable of observing dust and molecular line emission in star-forming complexes at high sensitivity and high angular resolution.

Fortunately, the advent of many-element millimetre-wave interferometers, such as those at Owens Valley, Hat Creek and Nobeyama Radio Observatory, should enable astronomers to locate a statistically significant sample of these 'missing links' in the star formation process. □

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REMOTE SENSING

Seeing earthquakes from afar

William Prescott

FOR anyone feeling jaded by overexposure to impressive but static satellite images of crinkled coastlines or mountain ranges, the new use of radar imagery demonstrated on page 138 of this issue may revive the sense of wonder. Massonnet and co-workers¹ use images taken by synthetic aperture radar to reveal the movement of the surface of the Earth. So detailed is the view of the deformation field that they have produced a 'map' of the displacement caused by last year's earthquake in Landers, California (see this week's cover).

This map may look familiar to some readers. We have seen similar maps showing the theoretical displacement field of earthquakes. But no other technique has been able to map the actual displacement field with anything like this kind of detail. The image contains information about the earthquake-produced motion of some 300 million points. For comparison, a study by Murray *et al.*² of this coseismic displacement field mapped the movement of just 28 points spread over the same area.

The displacement maps are produced by differencing radar images. A synthetic aperture radar mounted on an aircraft or satellite bounces a microwave signal off the ground, and the echoes are processed to produce an image of the ground surface. Each element of the image contains phase and amplitude information, but traditional radar images are a rendering of only the amplitude and mainly tell us the surface reflectivity at the wavelength used. In the late 1960s, investigators started to realize that the phase information was also valuable^{3–6}, and radar interferometry came into its own as a means of measuring topography⁷. For this, two separate images of the terrain taken from slightly different points are combined to produce a single image. The phase at each point in the combined image is equal to the difference in phase of the point in the two original images, and from these phase differences one can obtain the topography.

If two images could be taken from exactly the same point, the phase difference would be zero unless points in the image had moved. As in practice the images are never from precisely the same point, the effect of topography must be removed before movement of the surface can be detected. One approach is to use three images in all — two to obtain the topography, and a third, later image to find the displacement. Working along these lines, Gabriel and colleagues⁸ used three Seasat observations of the Imperial Valley in California to produce two 'interferograms'. Between the second and third observations, rainfall watered the ground, and the resulting swelling of the clay-rich soils by about 3 cm showed up clearly in the difference between the interferograms.

Massonnet and colleagues follow a different approach. They use a digital elevation model to remove topography and then map the changes from just two images, one taken before the earthquake and one after. The lateral resolution is a few centimetres.

Differential radar interferometry produces an estimate of the component of ground motion along the vector from the image point to the antenna, so images from three different directions would be required to map all three components of the displacement vector field of the surface. In principle, the precision can be a small fraction of the wavelength of the microwave carrier. In practice, it is limited by uncertainties in the position of the antenna, propagation effects, and changes in the reflectivity of the terrain. One of the biggest limitations is likely to be in correlating the phase when much time has elapsed between the images.

But Massonnet and co-workers have clearly shown that the technique works, and it could profoundly change the way geophysicists look at the deforming crust of the Earth. Until now, even modern techniques such as the Global Positioning System (where ground-based stations

obtain their position from satellite triangulation) yielded the deformation field at a few discrete points, each of which had to be visited by an observer. Opening the field to remote sensing would release a flood of new applications. We might inexpensively monitor all of the volcanoes in the world. Volcanic eruptions are often preceded by inflation of the volcanic edifices as magma moves into shallower reservoirs. At Mount Saint Helens, the bulge at Goat Rock became apparent even to the naked eye before the 1980 eruption. We could maintain a file of images of active faults, acquiring new data after an earthquake to produce accurate, up-to-date maps of the coseismic displacement field. We could look at ground subsidence due to water or oil withdrawal, monitor changes in glaciers, and detect landslides from a safe distance.

The high sampling density achieved is likely to lead to more profound and less predictable changes. One obvious application is in modelling the subsurface deformation field. We may be able to investigate the relation between the surface trace of faults and subsurface slip, or the variation of slip with depth on creeping faults such as the San Andreas or Hayward. There will, of course, be the non-uniqueness and resolution problems inherent in inferring any three-dimensional structure from a two-dimensional field. But the point density will provide information not previously available, particularly for shallow features. The images might swing the argument for or against disputed broad regional deformation patterns such as the Palm-dale bulge.

As well as the ERS-1 satellite images used by Massonnet and colleagues, observations could come from special-purpose aircraft flights or from NASA's Earth Observing System satellite, to be launched near the end of the century. Provided that the difficulties caused by 'uninteresting' changes (water content, vegetation, time of day, cloud cover) are not too serious, the impact of the technique could be huge. Interferometric radar imaging may not replace the many tools now used to study deformation of the Earth's surface, but it will certainly supplement them. □

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Coming soon — the movie

Ian P. Trayer

THREE new papers¹⁻³ provide us with a realistic snapshot of the contractile apparatus operating in muscle. This development has been long awaited, and now we can at last work towards producing the movie.

It is nearly a decade since the first reports of the crystallization of the head of the myosin molecule (S1) appeared. Since then the crystal structure of the actin monomer to atomic resolution has been solved¹ and modelled into the actin filament². In *Science*, Ivan Rayment and co-workers now report on the three-dimensional structure of the chicken

evidence for it. A wealth of data, however, too much to enumerate here, has established that the initial contact between actin and S1.ADP.P_i occurs in a weakly bound conformation that isomerizes to a more strongly bound form. This process is modulated by the status of the nucleotide bound to the myosin active site, particularly the presence or absence of the γ -phosphate. So the energy transduction step, coupling the hydrolysis of ATP to the movement of the filaments, must occur within the strongly bound states.

The new papers have implications that

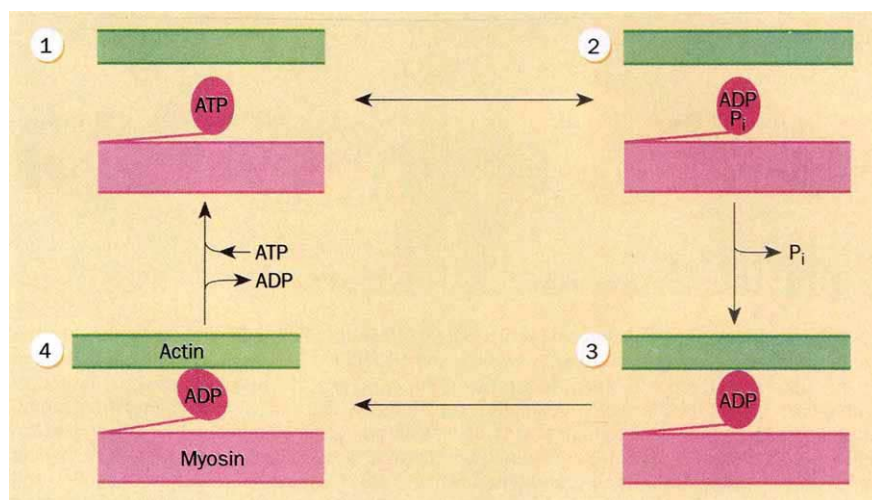


FIG. 1 Simplified scheme of the crossbridge cycle of muscle contraction. In resting muscle, a mixture of states 1 and 2, the myosin heads are able to interact with actin only weakly. The transition from states 2 to 3 is caused by the release of P_i from the myosin active site allowing the head to bind actin more tightly, change conformation and enter into the power stroke (from states 3 to 4). This is usually represented as a change in orientation of the head relative to actin, so pulling the filaments past one another. Each of the two heads on a myosin molecule (and all myosin molecules) are thought to cycle independently of one another.

skeletal myosin head³ and in two further papers, one accompanying the S1 crystal structure⁴ and one on page 171 of this issue⁵, the two crystal structures are combined with three-dimensional reconstructions from electron micrographs of frozen-hydrated F-actin decorated with S1 to provide an atomic model for the actomyosin complex.

Force generation in muscle occurs as the thin actin filaments and thick myosin filaments slide past one another as ATP is hydrolysed. The most widely accepted theory to explain this process involves the cyclic interaction of the myosin heads (the 'crossbridges') with actin, the heads changing their orientation with respect to the thin filament as a consequence of ATP binding, hydrolysis and product release. Indeed, this is the picture that has adorned most biochemistry and cell biology textbooks for many years (Fig. 1), although until very recently⁶ there was no hard

go far beyond muscle contraction. The interaction of actin, a highly conserved protein in evolutionary terms, with members of the myosin superfamily of proteins is responsible for force generation in a range of cellular processes, such as cytokinesis, vesicular transport and amoeboid movement. Indeed, cell biologists have coined the phrase 'molecular motors' to describe the protein (domains) that can perform contractile processes by moving cargoes along either actin filaments or microtubules. Each head of the two-headed muscle myosin (termed a type II myosin) represents a molecular motor. Other myosin IIs and a family of single-headed myosins (myosin Is), presumably looking rather like the S1 structure shown in Fig. 2 but with a variety of functionally specialized carboxy-terminal tails, occur in most non-muscle cells⁷. In the microtubule system, the kinesin and dynein families of molecular motors provide the

1. Massonnet, D. et al. *Nature* **364**, 138–142 (1993).
2. Murray, M. H., Savage, J. C., Lisowski, M. & Gross, W. K. *Geophys. Res. Lett.* **20**, 623–626 (1993).
3. Rogers, A. E. & Ingalls, R. P. *Science* **165**, 797–799 (1969).
4. Zisk, S. H. *Moon* **4**, 296–306 (1972).
5. Zisk, S. H. *Science* **178**, 977–980 (1972).
6. Rumsey, H. C., Morris, G. A., Green, R. R. & Goldstein, R. M. *Icarus* **23**, 1–7 (1974).
7. Zebker, H. A. & Goldstein, R. M. *J. geophys. Res.* **91**, 4993–4999 (1986).
8. Gabriel, A. K., Goldstein, R. M. & Zebker, H. A. *J. geophys. Res.* **94**, 9183–9191 (1989).

driving force for contraction.

It is now clear why it has taken so long from the first reports of the crystallization of S1 to the solving of the structure. The crystals of native S1 did not diffract to a high resolution despite much juggling with conditions and the authors were forced to rethink their strategy. Eventually they came up with the notion of reductively methylating all lysine residues to ϵ -dimethyllysine. It is likely that this stabilizes one of the (many?) conformations in which the molecules can exist and allows

structurally discrete domains. A key structural feature is the long α -helix (approximately 85 Å) that stretches from the thick part of the head to the carboxy terminus of the heavy chain around which the two light chains are 'clamped' in a protective manner. These closely resemble the way in which the Ca^{2+} -bound calmodulin interacts with its site on the smooth-muscle myosin light-chain kinase^{10,11}. (The myosin light chains, calmodulin and troponin C belong to the same family of Ca^{2+} -binding trigger pro-

mechanical devices in energy-transducing systems?

The other dominant structural feature is the cleft in the M_r 50K segment (red in Fig. 2) that extends from under the ATP site to the actin interface. Components of the actin-binding site lie on both the upper and lower domains of this cleft (Fig. 2) and it is suggested⁴ that this may open and close in response to the nature of the nucleotide in the active site. Not seen in this orientation of S1 is a fairly independent, small, six-stranded antiparallel β -sheet motif (similar to the Src-homology 3 domain) that protrudes from the 25K segment and could make contact with the next S1 along the actin filament^{4,5}. Interestingly, it is absent from many myosin I molecules.

The ATP enters phosphate first at a V-shaped depression about 13 Å deep on the opposite side of the molecule to the actin-binding sites. All three segments of heavy chain contribute to this site. The central strand of the dominant seven-stranded β -sheet corresponds to the strand-loop-helix β -phosphate binding motif seen in adenylate kinase and Ras protein. The S1 structure³ was determined without ATP but this motif and various affinity-labelling studies allow the site to be unambiguously identified. The binding of myosin to MgATP is very tight (binding constant $3 \times 10^{11} \text{ M}^{-1}$), which suggests that this cleft must close around the substrate. Such a structural change is suggested by the observation that the two reactive sulphhydryl groups, Cys 707 and Cys 697, lying on opposite sides of two α -helices in this region, can be crosslinked only in the presence of nucleotide¹³. If the ATP cleft does close, and the actin-binding face remains essentially stationary, then this could produce a movement in the carboxy terminus of the head (some 90 Å away from the ATP site) of approximately 60 Å (ref. 14).

In the image-reconstruction papers^{4,5}, we begin to see how the multi-site docking of the two proteins could explain the weak-to-strong binding transition that lies at the heart of the force-generation process. In order to prevent collisions between residues of actin and myosin in the docking, it is necessary to suppose that some conformational change on the myosin head and/or actin occurs. This is most easily accomplished by closing the cleft in the 50K segment⁴. The interactions between the two proteins comprise a series of ionic and ionic/hydrophobic contacts that are likely to occur sequentially to explain the kinetic and mechanical results. (One is reminded of the original Huxley and Simmons model¹⁵.)

The initial docking event is probably an ionic interaction between the highly basic loop connecting the 50K and 20K segments of S1 (a highly mobile region having no electron density) and acidic residues at

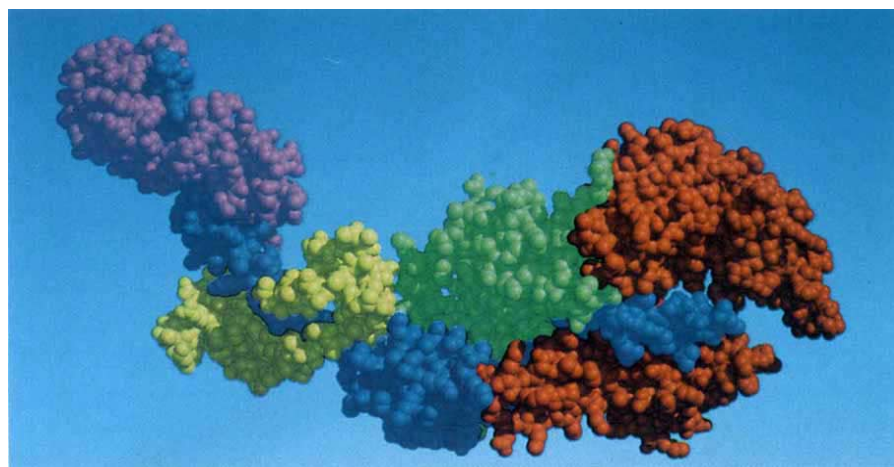


FIG. 2 Space-filling model of chicken fast-muscle S1 in which all amino acids are represented as alanine. The molecule is 165 Å long, 65 Å wide and 40 Å thick. The segments of the heavy chain are coloured green, red and blue and represent the regions of the heavy chain separable after limited proteolytic digestion with trypsin; the so-called 25K, 50K and 20K segments (from amino terminus to carboxy terminus) respectively. The essential (or alkali) light chain (yellow) and the regulatory light chain (magenta) are seen to be 'clamped' around the carboxy-terminal α -helical region of the heavy chain where it would join with the other myosin head to form the coiled coil rod that makes up the thick filament. In this orientation the prominent horizontal cleft that divides the central 50K segment of heavy chain into two domains (upper and lower defined by this orientation) is clearly visible. The actin-binding site is located primarily across this cleft at the lower right-hand corner. The entrance to the ATP site is shown as a depression in the amino-terminal 25K segment on top of the main bulk of the S1, although all three heavy-chain segments appear to contribute to the active site. (I. Rayment, from ref. 3.)

high-quality crystals to be produced. Whether or not this modifies the final structure in any serious way is open to question, but a vast array of solution work on S1 and acto-S1 can be explained by this structure and that proposed for the complex. Furthermore, it is clear that the methylated S1 behaves kinetically in the manner expected⁸ and that the structure of methylated lysozyme does not differ significantly from that of native lysozyme⁹. So one suspects that such gross chemical modification may now enter the crystallographers' arsenal of weapons when dealing with highly mobile proteins that prove difficult to crystallize.

The structure of S1 (Fig. 2) contains many surprising and interesting features. The main bulk of the head (where the green, red and blue segments overlap) comprises a seven-stranded β -sheet connected by flanking α -helices and/or loops. Each of the tryptic segments of the S1 heavy chain contribute to this, negating the long-held notion that they represented

teins, although in vertebrate myosins the light chains have lost their ability to bind Ca^{2+} specifically.)

This segment of heavy chain (blue in Fig. 2) extends along the whole molecule contributing to both the ATP site and the actin-binding sites, so directly connecting the functional end of the motor with the myosin filament. As we shall see, it is likely that this segment acts as the conformational coupler of the contractile process and this throws up a potentially exciting parallel with another energy-transducing system, the bovine heart mitochondrial ATPase¹². This ball-on-a-stalk shaped protein also contains a giant α -helix that runs from the base of the stalk, where it connects with the transmembrane proton gradient-generating F_0 subunit, through to the top of the ball (some 130 Å away) where the ATP-binding sites are located. Could it be that such long helices, protected from the solvent or stabilized by other protein subunits as necessary, act as simple

the amino terminus of actin. This would provide a weak interaction, sensitive to ionic strength, that would occur at long range and could be at a variety of orientations. The other interactions between this lower domain of the 50K segment and actin involve more stereospecific contacts between exposed hydrophobic residues on helix-loop-helix motifs on both actin and S1. On actin, the major contact region is the 'bottom' of subdomain 1 extending across the linking region between subdomains 1 and 3. It is also likely that there is contact with the 'top' of the subdomain 2 of the next actin monomer along the filament. A loop on the upper domain of the 50K segment, contacting a loop connecting the subdomains 1 and 3 of actin, appears to complete the primary contacts. A further region of weak electron density protrudes from the lower domain of the 50K segment and could also make contact with the next actin monomer down. Interestingly, this region is preserved in vertebrate myosins but contains deletions in some non-muscle myosins, so suggesting that a refinement of the interaction has evolved⁵.

Thus, the S1 appears to bind across two actins and penetrate well into the body of the actin filament. This indicates that a resurgence of the steric blocking model of the regulation of muscle contraction is on the cards, whereby tropomyosin could block one or more of these acto-S1 contact sites and still allow (unproductive) complex formation.

The most important implications of this model concern the interconnection between the opening and closing of the clefts in the 50K segment and at the ATP site. Binding of ATP would close the active site and open the 50K cleft, disrupting the actin-binding site and changing the angle by which the light-chain-clad tail is attached to the bulk of the head. After hydrolysis of the ATP, rebinding of the head to actin in a sequential multi-step process would lower the affinity of S1 for the γ -phosphate. Loss of this would trigger the power stroke and allow the S1 to reverse its conformational change induced by ATP binding. Thus, local and relatively small conformational changes at the actin

and ATP sites would lead to much greater distortions in the curvature of the tail. In other words, the bulk of the S1 would always bind to actin with about the same relative orientation but the tail would wag (cf. Fig. 1).

This model throws up some intriguing ideas. All conventional muscle myosins contain two sequential repeats of about 25 amino acids in the neck region of the molecule which we now know form an α -helix to which the light chains are clamped. This is the likely conformational coupler in the energy transduction step. The more unconventional myosin I molecules, on the other hand, can contain anything from one to six of these sequence motifs in their neck regions to which calmodulin(s) binds⁷. So the length of the

conformation coupler varies in these molecules and with it, one must suppose, the potential length of the power stroke per molecule of ATP hydrolysed. It is not yet clear from the S1 structure how closure of the ATP site would change the attitude of this tail to the bulk of the head, but any explanation must also take into account how modification of the light chains (for example by phosphorylation in smooth muscle or Ca^{2+} -binding in some invertebrate muscle myosins) can feed back to the ATP and actin-binding sites. There remains much to be done but at last we can begin to see where we are going. □

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COSMOLOGY

Lumps in the early Universe

John Peacock

COSMOLOGY has much in common with archaeology: just as it is hard to be certain how Stonehenge was erected given only the current state of the stones, so there is a variety of plausible theories for the origin of galaxies and their present-day large-scale clustering. The cure for the uncertainty is a time machine, and astronomers are now starting to amass data on the distribution of distant galaxies, seen when the Universe was young. The most popular theoretical predictions for this situation have assumed that the Universe is of high density, so that self-gravitating structures grow rather rapidly. This leads to the idea that structures such as clusters and superclusters of galaxies formed only recently. However, a new discovery by Francis and Hewett¹ suggests that this notion may be incorrect and that the distribution of galaxies when the Universe was a third of its present age was about as clumpy as it is today.

Francis and Hewett have observed not galaxies, but absorption lines due to clouds of neutral hydrogen seen in the spectra of high-redshift (distant) quasars. In a pair of quasars 8 arcminutes apart on the sky, they found two strong absorbers at somewhat different redshifts z around 2.5 ($1 + z$ gives the factor by which the Universe has since expanded), each of which was seen in both quasars. Such clouds are rather rare: the only plausible explanation for the observation is either that the clouds are so large that they span the distance between the lines of sight to the two quasars, or that the clouds are smaller objects which cluster very strongly on the scale of the separation probed here. The relevant number is a length which will have expanded by the present to 6 megaparsecs (assuming a high-density Uni-

verse, and the largest plausible Hubble constant). For comparison, the typical separations between bright galaxies are about 1 megaparsec.

The idea that the high-redshift Universe contains extended clouds of neutral hydrogen might seem attractive in view of the claimed detection by Uson *et al.* of one such cloud in 21-cm microwave emission^{2,3}, with a similar size to that required here. On the other hand, this raises the question of what the clouds might be. The size suggests galaxy-cluster or supercluster scales, but the only models in which these structures start life with a large neutral-hydrogen content are 'pancake' theories where the Universe contains no smaller structures. Galaxies then form via fragmentation in dense sheets — which is hard to reconcile with a variety of evidence, such as the existence of galaxy systems of low density contrast like the local group that includes the Milky Way. In any case, the cloud cannot have anything like a smooth structure: the column densities of hydrogen in front of the two quasars differ by a factor of 100. This sounds more like a group of discrete sub-clouds. Finally, it seems⁴ that other observers cannot reproduce the detection by Uson *et al.* (P. van der Kruit, personal communication).

We are left with the idea that the clouds are related to galaxies, which must be strongly clustered. Francis and Hewett estimate that an enhancement in density of a factor of about 30 is required, which must thus extend over a sphere of at least 3 megaparsecs radius. In the Universe today, the fractional root-mean-square density fluctuation between different spheres of this size is roughly 2.5. Of course, galaxies are not random points but

1. Kabsch, W., Mannherz, H. C., Suck, D., Pai, E. & Holmes, K. C. *Nature* **347**, 37–44 (1990).
2. Holmes, K. C., Popp, D., Gebhard, W. & Kabsch, W. *Nature* **347**, 44–49 (1990).
3. Rayment, I. *et al.* *Science* **261**, 50–58 (1993).
4. Rayment, I. *et al.* *Science* **261**, 58–65 (1993).
5. Schroder, R. R. *et al.* *Nature* **364**, 171–174 (1993).
6. Irving, M., Lombardi, V., Piazzesi, G. & Ferenczi, M. *Nature* **357**, 156–158 (1992).
7. Cheney, R. E. & Mooseker, M. S. *Curr. Opin. Cell Biol.* **4**, 27–35 (1992).
8. White, H. D. & Rayment, I. *Biochemistry* (in the press).
9. Rypniewski, W. R., Holden, H. M. & Rayment, I. *Biochemistry* (in the press).
10. Ikura, M. *et al.* *Science* **256**, 632–638 (1992).
11. Meador, W. E., Means, A. R. & Quijcho, F. A. *Science* **257**, 1251–1255 (1992).
12. Abrahams, J. P. *et al.* *EMBO J.* **12**, 1775–1780 (1993).
13. Wells, J. A. & Yount, R. G. *Meth. Enzymol.* **85**, 93 (1992).
14. Wakabayashi, K. *et al.* *Science* **258**, 443–447 (1992).
15. Huxley, A. F. & Simmons, R. M. *Nature* **233**, 533 (1971).

define the high-density regions; it is easy to show that the mean fractional density enhancement around a given galaxy is just the square of the root-mean-square value, so a factor of 30 would be atypically high even at the present epoch. This would seem to indicate that clustering at high redshifts cannot easily be significantly weaker than that today, and other lines of evidence point in this direction. Wolfe has shown⁴ that the absorption clouds discussed here have an enhanced density of line-emitting neighbours at $z = 2.6$ — by at least a factor of 11.6 within a radius of roughly 3 megaparsecs. Studies of the clustering of quasars also show little evolution of their correlation amplitude between $z = 2$ and the present⁵.

There are two obstacles to accepting this picture. One is theoretical: as already mentioned, high-density universes should have much lower density fluctuations at high redshift (the scale decreases as $1/(1+z)$). Although distasteful to many theorists, low-density models will be much more strongly clumped at high redshift, and would be favoured if the above evidence was all we had to go on. However, there are strong indications from the weak clustering of faint galaxies seen at optical wavelengths that galaxy clustering does indeed evolve at close to the above high-density rate⁶. The problem is to decide which of the three sets of objects (faint galaxies, gas clouds, quasars) most faithfully reflects the behaviour of the underlying mass distribution. This is not a simple task, as it requires an understanding of how each class of object forms, and a case can be made in favour of either high or low densities. If the density was high, we would say that faint galaxies roughly trace mass, whereas the massive galaxies associated with quasars and absorption-line clouds display clustering that is largely unevolving statistical bias⁷. Alternatively, the high-redshift faint blue galaxies might be a distinct population with intrinsically weak clustering.

Much thought is being devoted to this problem, and we may hope that the present state of slight confusion and contradiction will not persist. In any case, it is clear that projects to map the distribution of matter at high redshifts will be one of the major areas of cosmological effort in coming years. □

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Expanding the potential

Richard Morris and Graham Collingridge

LIFE is a gas. Hard on the heels of all the excitement surrounding the discovery of nitric oxide as an intercellular messenger in the central nervous system¹, come two reports, one in *Science*² and one on page 147 of this issue³, which point to the involvement of another gas, carbon monoxide, in triggering synaptic plasticity. The interest of these new papers lies largely in how the results they describe bear on the mysterious phenomenon of long-term potentiation.

Long-term potentiation (LTP) is a widely studied form of activity-dependent synaptic plasticity which may underlie learning and memory. One question of especial interest is how the mechanisms of LTP induction, which start with activation of *N*-methyl-D-aspartate (NMDA) receptors located within postsynaptic neurons, can communicate with presynaptic terminals. Such communication seems to be required, given that the expression of an increased synaptic response is thought to be partly presynaptic⁴.

An intriguing candidate agent came to light with the discovery that stimulation of NMDA receptors can lead to the production and release of nitric oxide (NO) from neurons¹. This gas has several of the properties appropriate for a retrograde messenger — particularly its ability to diffuse rapidly and its short half-life. In support of this proposal, there have been several indications that NO is involved in LTP^{5–8}. Most notably, both inhibitors of NO synthase and scavengers of NO itself can block its induction. These results are not universally obtained, however, and there has been concern about the low levels of expression of NO synthase within the pyramidal neurons of the hippocampal formation⁴.

Zhuo *et al.*², and Stevens and Wang³, now present evidence implicating a different gas, carbon monoxide, in synaptic plasticity. Both groups report that zinc protoporphyrin IX (ZnPP) — an inhibitor of haem-oxygenase, the enzyme that makes carbon monoxide (CO) in the brain — blocks the induction of LTP. Strikingly, Stevens and Wang also find that ZnPP erases pre-established LTP.

The revelation that CO is a suspect for the long-sought retrograde messenger bears upon certain of the concerns raised about NO. Immunocytochemical studies have, for example, established that haem-oxygenase is present in appreciable amounts within hippocampal pyramidal cells. In addition, the observation that haemoglobin blocks LTP is equally compatible with either NO or CO serving the function of an intercellular messenger, as haemoglobin actively binds both gases.

But the champagne should be kept on ice for a while yet; it would be premature to conclude that CO is indeed the retrograde messenger in LTP. First, neither set of authors presents data to show that ZnPP, which works successfully in cell cultures⁹, actually inhibited CO formation within the brain slices in which LTP was blocked. Second (and more of a worry) is that little is known about the specificity of ZnPP as an inhibitor. In this context, it has been shown that ZnPP blocks soluble guanylate cyclase¹⁰. A further necessary control, yet to be carried out, is to explore the effects of copper protoporphyrin IX which is relatively inactive in inhibiting haem-oxygenase. Third, even assuming that ZnPP inhibits CO in brain slices, and in a specific manner, its mechanism of inhibiting LTP remains unclear. Carbon monoxide might be functioning as an intercellular messenger and, in support of this, Zhuo *et al.*² show that coupling CO with synaptic activity induces LTP. This potentiation occurs even in the presence of an NMDA antagonist, suggesting that CO works downstream of these receptors. However, there is a problem in the activation of haem-oxygenase. At present there is no evidence that this enzyme is stimulated by NMDA or regulated by calcium.

So, what else could be happening? An alternative mechanism is suggested by the work of Glaum and Miller¹¹, and is that ZnPP blocks many of consequences of the stimulation of metabotropic glutamate receptors. The significance of this other effect of ZnPP, which may or may not involve CO, is that activation of these receptors is necessary for the induction of LTP¹².

Several formal criteria would need to be met before CO could be accepted as a retrograde messenger. One important conceptual conundrum arises from the observation that application of ZnPP

1. Francis, P. J. & Hewett, P. C. *Astr. J.* **105**, 1633–1636 (1993).

2. Uson, J. M., Bagri, D. S. & Cornwell, T. J. *Phys. Rev. Lett.* **67**, 3328–3331 (1991).

3. Peacock, J. A. *Nature* **355**, 203–204 (1992).

4. Wolfe, A. M. *Astrophys. J.* **402**, 411–419 (1993).

5. Andreani, P. & Cristiani, S. *Astrophys. J.* **398**, L13–L16 (1992).

6. Couch, W. J., Jurcevic, J. S. & Boyle, B. J. *Mon. Not. R. astr. Soc.* **260**, 241–252 (1993).

7. Cole, S. & Kaiser, N. *Mon. Not. R. astr. Soc.* **237**, 1127–1146 (1989).

1. Garthwaite, J., Charles, S. L. & Chess-Williams, R. *Nature* **336**, 385–388 (1988).

2. Zhuo, M., Small, S. A., Kandel, E. R. & Hawkins, R. D. *Science* **260**, 1946–1950 (1993).

3. Stevens, C. F. & Wang, Y. *Nature* **364**, 147–149 (1993).

4. Bliss, T. V. P. & Collingridge, G. L. *Nature* **361**, 31–39 (1993).

5. Bohme, G. A. *et al.* *Eur. J. Pharmacol.* **199**, 379–381 (1991).

6. O'Dell, T. J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **88**, 11285–11289 (1991).

7. Schumann, E. M. & Madison, D. V. *Science* **254**, 1503–1506 (1991).

8. Haley, J. E. *et al.* *Neuron* **8**, 211–216 (1992).

9. Verma, A. D. J., Giatt, C. E., Ronnett, G. V. & Snyder, S. H. *Science* **259**, 381–384 (1993).

10. Ignarro, L. J., Ballot, B. & Wood, K. S. *J. biol. Chem.* **259**, 6201–6207 (1984).

11. Glaum, S. R. & Miller, R. J. *Molec. Pharmacol.* **43**, 965–969 (1993).

12. Bashir, Z. *et al.* *Nature* **363**, 347–350 (1993).

13. Barnes, C. A. in *Dahlem Konferenzen Life Sciences*, Vol. 54 (in the press).

14. Morris, R. G. M. *et al.* *Nature* **319**, 774–776 (1986).

15. McLaughlin, J. L. *Science* **153**, 1351–1358 (1966).

erases pre-established LTP. If CO were acting as a retrograde messenger, one might expect its action to be limited to the time shortly after the brief high-frequency stimulus used to induce LTP. The CO released would then trigger some biochemical cascade within proximal presynaptic terminals, leading to increased transmitter release. But Stevens and Wang³ observed that ZnPP could erase LTP in a potentiated pathway, without affecting a control pathway, even when applied 30 minutes after induction. This remarkable finding means that CO could be enabling some tonically active process that is necessary for the expression of LTP.

Whatever the mechanism, the possibility that LTP can be erased pharmacologically is potentially of considerable value in the analysis of LTP's functional significance. To date, there are two main lines of evidence that the underlying mechanisms of LTP are activated during, and are required for, certain kinds of learning — first, from studies showing that certain physiological properties of LTP (such as decay time course) covary with indices of behavioural learning¹³; and

second, that the blockade of hippocampal LTP *in vivo* is associated with a selective impairment of hippocampal-dependent learning¹⁴. Fresh points of attack would be helpful.

One such approach, suggested by Stevens and Wang's findings, would be to explore whether ZnPP can selectively erase recent memories. Provided ZnPP erased LTP *in vivo*, its application shortly after a learning experience would obviate certain interpretive problems associated with giving drugs before training episodes¹⁵; that's because there would then be no possibility that the drug was interfering with perceptual or motor processes during learning itself. Finding that ZnPP could cause animals to forget would be memorable, and such a result would further potentiate the hypothesis that LTP underlies learning. □

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ATMOSPHERIC SCIENCE

Ideas flow on Antarctic vortex

William Randel

OZONE destruction within the vortex that forms over the Antarctic during polar winter gives us the all too familiar 'ozone hole'. But if large volumes of air are swept through the vortex, ozone-depleting reactions at the poles could also be affecting mid-latitudes, where most of the world's population lives. New results from the Upper Atmosphere Research Satellite (UARS), presented at a conference in late May*, have rekindled arguments over just how fast material does flow through the stratospheric vortex.

The controversy centres on whether air is effectively contained on seasonal time-scales within the vortex, horizontally by the polar night jet stream and vertically by weak vertical velocities¹, or whether there is relatively rapid air transit through the system². High in the atmosphere (more than 40 kilometres up) there appears to be rapid downward motion into the vortex, associated with convergence of the mean summer to winter hemisphere flow in the mesosphere. But as density decreases exponentially with height, this strong inflow can be balanced by modest flow out of the vortex in the lower stratosphere (see figure overleaf).

The degree of flow-through is important for understanding the budgets of trace constituents within and outside the

vortex (and its Northern Hemisphere counterpart). Fast flow-through implies continual refreshing of air within the vortex, so that chemical processing (such as that responsible for the Antarctic ozone hole) would be substantially faster than would be the case for isolated air. Moreover, it would mean that air outside the vortex over much of the hemisphere had experienced the intense cold and chemically perturbed conditions within the vortex and the effects would be propagated to middle and low latitudes. In this case, the vortex is termed a 'flowing processor'.

This latter signature is the basis for the suggestion that UARS data show a flowing processor. Observations of water vapour and methane from the Halogen Occultation Experiment (HALOE) on UARS indicate substantial dehydration within the polar vortex (A. Tuck, NOAA Aeronomy Laboratory). This occurs each winter in the Antarctic, as water vapour is frozen out in the intense cold of the vortex interior. The intriguing observation from HALOE is that the dehydration signature extends far into middle and low latitudes, over a deep layer of the lower stratosphere (16–24 kilometres). Because temperatures cold enough to freeze out stratospheric water vapour are found only within the Antarctic vortex and at this time of year, the widespread dehydration sug-

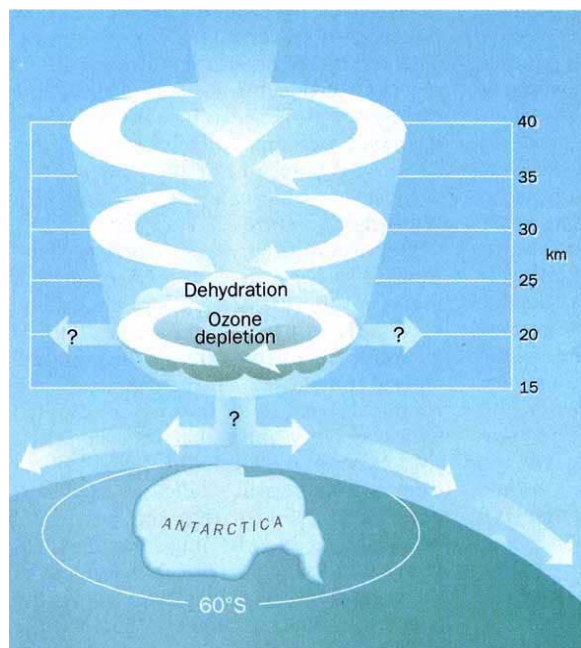
gests that air over much of the hemisphere has recently traversed the vortex interior. The volume of air in the vortex is 10–20 per cent of that for the rest of the dehydrated hemisphere, so it looks like there has been a complete exchange of vortex air several times during the winter; Tuck suggests a timescale of 30 days for 'flushing' the vortex.

Not everyone agrees. Opponents of the flowing processor concept argue that there is little mass transport out of the vortex. They have a formidable body of evidence for this — aircraft and satellite measurements of the dynamical and chemical structure of the vortex, coupled with trajectory and radiative calculations (M. Schoeberl, Goddard Space Flight Centre). Numerical studies of such vortices back the idea that the horizontal boundary is nearly impermeable³ (as seems intuitively likely from the observed steep gradients of constituents across the edge). Simulations suggest that a small amount of material is peeled off the outer edge of the vortex in narrow tongues or filaments over the course of the winter, and this material is then rapidly mixed over mid-latitudes. High-resolution simulations of this process in the real atmosphere, based on trajectory calculations, agree well with satellite- and aircraft-based constituent measurements, and confirm the earlier findings of low material flux from inside the vortex to outside (D. Waugh and A. Plumb, Massachusetts Institute of Technology, and B. Pierce, NASA Langley Research Center).

Furthermore, analysis of trace constituent fluxes mapped at high resolution from aircraft has shown that horizontal transport across the vortex on smaller spatial scales (down to 10 kilometres) is insignificant⁴. So it appears that net flow through the sides of the vortex is minimal, although there is probably a good deal of mixing between the vortex edge and air well away from the poles.

This leaves transport out of the bottom of the vortex as the other possible mechanism for flushing (although it is not obvious how such transport could be communicated back to the mid-latitude lower stratosphere). To flush the whole of the vortex volume over the 16–24-kilometre layer of the lower stratosphere with a 30-day timescale requires a vertical velocity of about 0.1 cm s⁻¹ averaged over the area of the vortex at a height of 16 kilometres. The vertical velocity of air parcels is a difficult quantity to measure. A convenient substitute for height that can be readily measured from aircraft is the 'potential temperature' of the air, which is the air temperature adjusted for adiabatic expansion effects. Recast in these terms, the downward velocity near 16 kilometres is equivalent to a potential temperature increase at a rate of just 1.2–1.4 kelvin per day. In equilibrium,

*American Geophysical Union Meeting, Baltimore, 24–28 May 1993.



The Antarctic polar stratospheric vortex in middle to late winter; the vertical scale is exaggerated by a factor of about a hundred. The vortex is defined by the stratospheric jet-stream winds circulating about the South Pole (with maximum speeds near 100 m s^{-1} in the upper stratosphere in mid-winter), and by the associated horizontal gradients of potential vorticity¹⁻³. Air flows strongly downwards into the vortex above 40 km. The rate of horizontal and vertical flux out of the vortex below 25 km is the focus of the debate over whether the vortex is a 'flowing processor' or a 'containment vessel'. Dehydration and ozone depletion both occur in the lower stratosphere at heights between about 15 and 25 km. Both are ultimately due to the intense cold within the vortex at this height (below -80°C): dehydration results directly from freeze-out of water vapour, ozone depletion from chlorine-catalysed processes which in turn occur primarily in the presence of polar stratospheric clouds (composed of ice or hydrates of nitric acid).

this tendency would be balanced by infrared radiative cooling of the lower stratosphere.

Do the measurements bear this out? Preliminary estimates of these descent rates from the HALOE data, based on the vertical displacements of the dehydrated region within the vortex during one week in mid-October (after temperatures have risen above the frost point) look promising (1.7 ± 0.3 kelvin per day, near 19 kilometres; Tuck). But extensive aircraft observations of the long-lived tracer N_2O in the Antarctic vortex during August and September 1987 showed little vertical variation with time, suggesting cooling rates less than 0.2 kelvin per day^{5,6}. Furthermore, similar small values are derived from independent calculations of the radiative cooling rates in the polar lower stratosphere^{7,8} (which, as noted above, must balance the descent rates in equilibrium).

The difference between the rival descent rates may be attributable to the shorter time record analysed by HALOE, together with the fact that vortex descent

rates are highest during late spring (October). But it seems difficult to reconcile the earlier N_2O observations and current radiative transfer calculations with the strong flow out of the bottom of the vortex needed throughout the entire winter to produce the mass flux proposed by Tuck.

The HALOE observations of hemispheric-scale dehydration in the Southern Hemisphere present atmospheric scientists with an intriguing problem. Matters may become clearer with further analyses of a new general circulation model simulation of the stratospheric water vapour budget, which shows mid-latitude dehydration in the southern stratosphere similar to that observed by HALOE⁹ (although the transport mechanism remains to be established).

Transport of vortex air to mid-latitudes would leave other clues to its presence. Decreasing concentrations of ozone have been observed over mid-latitudes of both hemispheres, and this as yet unexplained observation may be a signature of vortex processing. This may be more likely in the Northern Hemisphere stratosphere¹⁰, where the

downward circulation in the vortex is stronger than that in the south. Although dehydration is not widespread in the Northern Hemisphere vortex because the air is too warm, the CLAES and MLS instruments, also on board UARS, have been looking for tell-tale signs of vortex processing in the concentrations of other trace gases. When the results are analysed in detail, we can expect fresh contributions to the debate. □

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1. Schoeberl, M. E. *et al.* *J. geophys. Res.* **97**, 7859–7882 (1992).
2. Tuck, A. F. *J. geophys. Res.* **94**, 11687–11737 (1989).
3. Juckes, M. N. & McIntyre, M. *Nature* **328**, 590–596 (1987).
4. Bacmeister, J. T. & Schoeberl, M. R. *Geophys. Res. Lett.* **19**, 1101–1104 (1992).
5. Hartmann, D. *et al.* *J. geophys. Res.* **94**, 16779–16796 (1989).
6. Lowenstein, M. *et al.* *J. geophys. Res.* **94**, 11589–11598 (1989).
7. Shine, K. Q. *J. R. met. Soc.* **115**, 265–292 (1989).
8. Rosenfield, J. *et al.* *J. Atmos. Sci.* **44**, 859–876 (1987).
9. Mote, P. W. *et al.* *Geophys. Res. Lett.* (in the press).
10. Stolarski, R. S. *et al.* *Science* **256**, 342–349 (1992).

DAEDALUS

Outfacing time

'YOUTH's a stuff will not endure', at least facially. The encroaching lines and wrinkles of maturity fill many of us with dismay. Some of us are so dismayed that we even seek surgical help.

In the standard face-lift, the surgeon removes a chunk of skin and stretches the rest to fill its place, thus ironing out the wrinkles. The procedure may seem rather drastic, but has nature on its side. In the normal process of wound-healing, the dermis contracts around the region of damage; so the surgeon can rely on a useful automatic reduction of skin area. Even so, the removal of one big chunk of skin, and the stretching of the rest, tends to distort the patient's features. One way of avoiding this might be to remove very many small areas of skin, distributed evenly over the region to be smoothed. Daedalus is now taking the idea to extremes. He proposes to remove millions of micron-sized regions of dermis, each too small to see.

He has been inspired by the technology of grit-blasting with solid carbon dioxide. It is surprisingly gentle. The tiny particles chip away dirt and dust from the object to be cleaned, and then evaporate perfectly to leave no residue. Solid carbon dioxide seems a bit drastic as a facial abrasive, but micron-sized pieces of ice (or better, frozen medical saline solution) should do the trick nicely. So the DREADCO Wrinkle Blaster is a sort of small-scale thunderstorm. It grinds ice into many tiny particles, and charges and accelerates them in a big electric field. The resulting miniaturized hailstorm is directed onto the subject's skin. Each particle penetrates a fraction of a millimetre, just sufficient to traverse the epidermis and enter the dermis. (There is a ballistic immunizer that works this way.) The ice melts and the microscopic lesion soon heals, with the normal contraction of dermal healing. The skin is fractionally tightened at that point, and the epidermis reforms scarlessly over the tiny wound. The millions of tiny pinpricks contract the whole region of skin, evenly and strongly.

Thus the facial clock will be put back, and the cruel marks of time erased. Wrinkle Blasting will certainly sting severely, but may not need a local anaesthetic. Daedalus even hopes that the patient can control the whole treatment. DREADCO could then market the Wrinkle Blaster as a consumer product, for private facial maintenance and editing. Wrinkles may best be blasted in many small, controlled stages. The resulting slow and subtle facial rejuvenation will then arouse appreciation but no suspicion in friends, associates and lovers. David Jones

Denticles in thelodonts

SIR — Thelodonts are the most enigmatic of all Palaeozoic jawless vertebrates, because their internal skeleton was not ossified and their exoskeleton was made up of minute scales which were often dispersed during decay. Scotland is one of the few places where articulated thelodonts are found, where they occur in 425-million-year old Silurian rocks. Acid preparation carried out by W. V. B. on some well-preserved Scottish thelodonts has yielded for the first time peculiar internal assemblages of minute denticles or denticle-bearing plates, which recall some of the pharyngeal dermal elements of jawed vertebrates and were hitherto unrecorded in any jawless vertebrate. Some of these thelodont dermal elements have recently been mentioned¹⁻³, but we are now able to describe their precise distribution inside the animal, and provide a possible explanation of their function.

The internal denticles fall into three types: (1) located inside the snout, consisting of minute, forward-pointing denticles (*a* in the figure); (2) located further back in the centre of the head, consisting of somewhat similar, but backward-pointing denticles (*b*); and (3) located near the

presumed branchial openings, consisting of slender cusped denticles, often fused into thin, curved series arranged side by side (*c*). This discovery of an extensive exoskeletal covering in the mouth and pharynx of a jawless vertebrate raises several questions. Did other Palaeozoic jawless vertebrates, in particular the armoured forms, or 'ostracoderms', also possess such internal denticles, hitherto unnoticed? Or were some thelodonts more closely related to jawed vertebrates, as recently suggested on other grounds^{4,5}?

The denticles of the second and third type can easily be interpreted as pharyngeal denticles or tooth whorls associated with the gill bars or with the extrabranchial ducts. Similar tooth whorls occur in Palaeozoic sharks, for example⁶. More puzzling are the forward-pointing denticles in the snout, which cannot be regarded as buccal denticles or teeth because of their orientation. However, similar forward-pointing denticles or tubercles are observed on the wall of the large median dorsal duct of galeaspid (a group of Palaeozoic armoured jawless vertebrates⁵), which was for intake of respiratory water. The role of such struc-

tures, directed against the water flow, may have been either to repel ectoparasite larvae or to convey part of the water flow toward the nasal cavities. If this analogy is correct, thelodonts may have had some kind of large terminal inhalent duct, comparable in structure and function to that of extant hagfishes, a condition which is currently regarded as primitive for vertebrates, but lost in lampreys and jawed vertebrates⁵. The co-existence of such a large median inhalent duct connected with the nasal sacs, and pharyngeal denticles or tooth whorls, are what would be expected in a theoretical pre-gnathostome.

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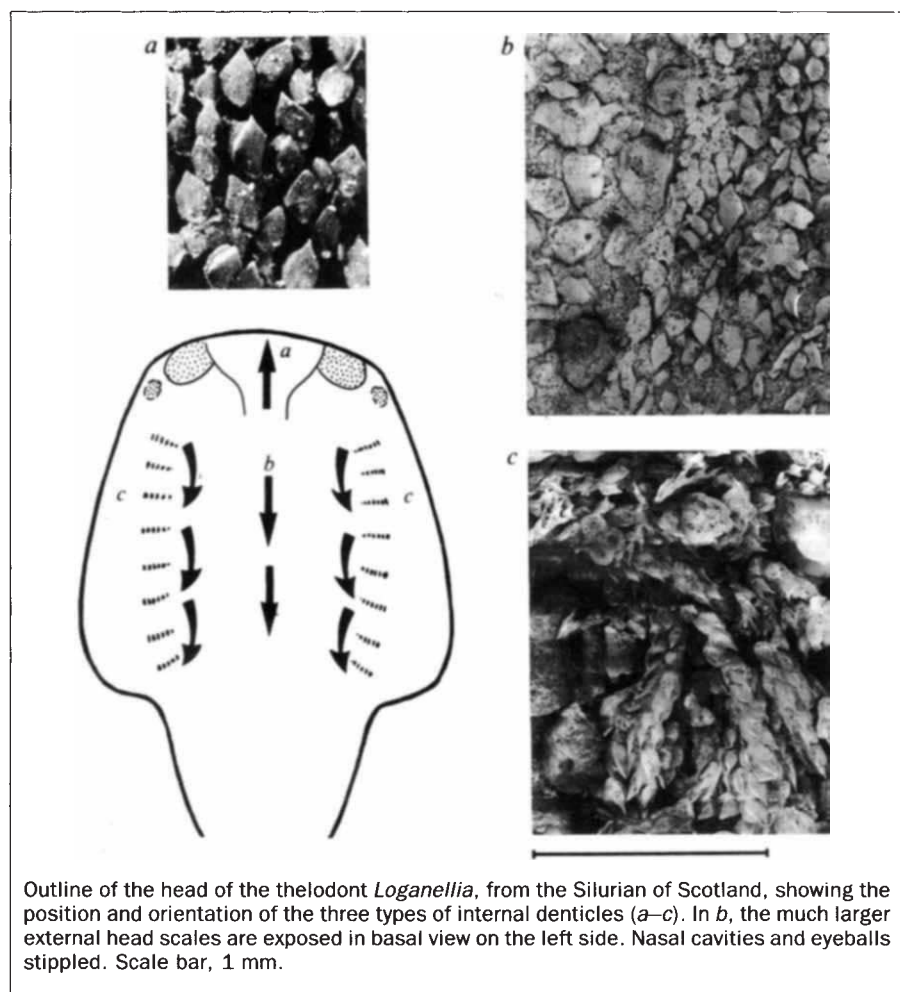
1. Van der Bruggen, W. *Ichthyolith Issues* **9**, 7-9 (1992).
2. Vergoosen, J. M. J. *Geologie en Mijnbouw* **71**, 51-64 (1992).
3. Turner, S. in *Early Vertebrates and Related Problems of Evolutionary Biology* (eds Chang, M. M., Liu, Y. H. & Zhang, G.) 87-119 (Science Press, Beijing, 1991).
4. Wilson, M. V. & Caldwell, M. W. *Nature* **361**, 442-444 (1993).
5. Forey, P. & Janvier, P. *Nature* **361**, 129-134 (1993).
6. Maisey, J. G. *Am. Mus. Novitates* **2931**, 1-42 (1989).

Protein structure and introns

SIR — In the view of Gilbert^{1,2} and others, genes encoding modern proteins arose by the intron-mediated assembly of ancestral RNA genetic units encoding protein structural motifs. A recent boost for this model came from the discovery in the triose phosphate isomerase (TIM) gene of a mosquito³ of a novel intron mapping to a position predicted several years earlier⁴. This is interpreted to be a rare survivor of an intron which was present in the assembly of an ancestral TIM gene billions of years ago, an intron which was generally lost in most descendant species. There is, however, a very simple alternative explanation for mapping between intron position and protein structure which requires neither the invocation of coincidence nor an ancient, gene-assembly origin for introns.

It is widely believed that, very early on, RNA was used as both genetic storage material and cellular effector molecule. Labile RNA 'genes' might have enhanced their stability by adopting folded tertiary structures, capable of being unfolded during translation. As in the Gilbert model, these genes would have encoded small protein structural motifs able to associate in various combinations to form multisubunit complexes, equivalent to modern proteins, with selectable functions.

Complex continuous genes could then



Outline of the head of the thelodont *Loganellia*, from the Silurian of Scotland, showing the position and orientation of the three types of internal denticles (*a-c*). In *b*, the much larger external head scales are exposed in basal view on the left side. Nasal cavities and eyeballs stippled. Scale bar, 1 mm.

have arisen by direct ligation of these ancestral RNA genetic modules. Such fusion would have improved the efficiency of protein domain assembly. Consequently both RNA and encoded proteins would have had a structural mapping, with more or less discrete modules strung together by connecting loops and a direct correspondence between protein and RNA motifs.

If introns subsequently invaded the genome with an ability to 'self-splice' into RNA⁵, those regions most susceptible to insertion could well have included the relatively unstructured connecting loops between RNA domains. Introns could thus have been preferentially directed to positions corresponding to protein structural divisions. Different RNA molecules would have had different degrees of structure and different abilities to direct insertion. There would even have been a tendency for introns to insert at similar, but not identical, locations in different lineages, giving the appearance of intron slippage. As in other models, introns would have arrived in the DNA genome by reverse transcription, perhaps using intron⁶ or retrovirus-encoded enzymes.

The selective advantage of discrete folding in coding sequences would probably have declined as riboprotein complexes and other systems evolved to handle RNA. However, the need to maintain encoded protein sequence and the benefits of increased stability could have acted to retain ancestral RNA structure for some time. Even now, some degree of internal folding in transcribed exons could be advantageous in improving the energetics of splicing.

In summary, the positions of many introns may truly be nonrandom. However, their positioning need not mean that ancient introns participated in the original assembly of genes. Introns could instead have been inserted at later times with their positions determined by how well ancestral RNA structure was retained. The successful prediction of a novel intron position in TIM may thus be the result of an insightful analysis of protein structure rather than a validation of the idea of ancient, intron-mediated gene assembly.

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Life expectancy and reproduction

SIR—Recent developments in life-history theory predict that organisms will alter reproductive behaviour if life expectancy declines¹⁻³. Here we report that a parasitic wasp responds to changes in barometric pressure associated with thunderstorms, which are known to cause considerable mortality among small, frail insects⁴.

Parasitic wasps usually do not lay eggs in low-quality (already parasitized) hosts; this is an adaptive behaviour for maximizing lifetime reproduction^{2,3}. We predicted³ that wasps will oviposit in such poor hosts if the gain in lifetime reproduction associated with acceptance is higher than it would be for rejection. This trade-off is determined in part by the availability of hosts (habitat quality) and in part by life expectancy, which can change very suddenly with the approach of storms. The two main predictions of the theory³ are that a wasp will spend more time on a patch of previously parasitized hosts and will superparasitize (lay an egg in a previously parasitized host) more often if the risk of mortality increases.

To manipulate the perceived quality of

the habitat, we raised the parasitic wasp *Leptopilina heterotoma*⁵ on host patches with only unparasitized fruit fly larvae (good world, G) or only parasitized larvae (bad world, B) (see ref. 3 for explanation). To manipulate perceived life expectancy, we conducted the experiment under steady barometric pressure (S) as would occur during a fair summer day or rapidly dropping barometric pressure (D) as would occur several hours before the onset of a large storm. We placed wasps and patches containing only parasitized hosts in sealed plastic chambers, which were then connected to a microprocessor-driven air pump controlling internal air pressure. At the start of the experiment, we randomly chose the boxes to correspond to the steady pressure or dropping pressure, which decreased at 1 mbar per hour. Six hours later, we released the wasps individually on to the patches and recorded times on patches and number of superparasitisms.

The wasps are sensitive to barometric pressure in the manner predicted by theory. Wasps in the low life-expectancy treatments searched longer ($P < 0.02$; part *a* in the figure), encountered more hosts and superparasitized more frequently ($P < 0.00014$, part *b*). Explanations other than decrease in life expectancy are possible, but our previous work³ shows that wasps respond in a similar manner to a completely different cue (photoperiod) that indicates short life expectancy. Hence it is life expectancy and not the cue that is shaping reproductive behaviour. We cannot firmly state that the wasps respond only to dropping barometric pressure or to changes in barometric pressure. However, the point could be moot as it may be the indication of change (and the uncertainty associated with such change) that has shaped the behaviour of the wasps.

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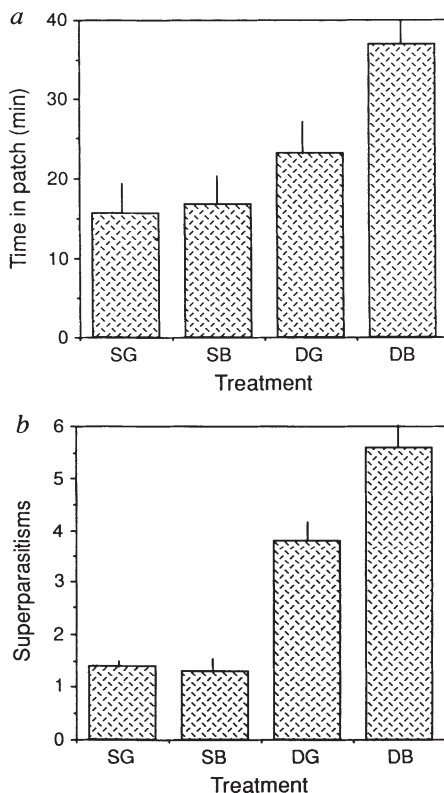
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a, Amount of time wasps allocated to searching patches before emigrating. SG, steady pressure, good world; SB, steady pressure, bad world; DG, dropping pressure, good world; DB, dropping pressure, bad world. *b*, Number of times wasps superparasitized. Data are shown with $\pm$ standard error.

- Gilbert, W., Marchionni, M. & McKnight, G. *Cell* **46**, 151–153 (1986).
- Dorit, R. L., Schoenbach, L. & Gilbert, W. *Science* **250**, 1377–1382 (1990).
- Tittiger, C., Whyard, S. & Walker, V. K. *Nature* **361**, 470–472 (1993).
- Marchionni, M. & Gilbert, W. *Cell* **46**, 133–141 (1986).
- Sharp, P. A. *Cell* **42**, 397–400 (1985).
- Michel, F. & Lang, B. F. *Nature* **316**, 641–643 (1985).

- Mangel, M. & Clark, C. W. *Dynamic Modeling in Behavioral Ecology* (Princeton University Press, 1988).
- Mangel, M. & Ludwig, D. A. *Rev. ecol. Syst.* **23**, 507–536 (1992).
- Roitberg, B. D. *et al. Behav. Ecol.* **3**, 156–165 (1992).
- Wellington, W. G. *Can. J. Res. Sec. D24*, 51 (1946).
- Visser, M., van Alphen, J. J. M. & Nell, H. W. *Behavior* **114**, 21–36 (1990).

Infiltration of mariner elements

SIR — Robertson reports¹ the presence of different families of *mariner* transposon elements² in many insects using a polymerase chain reaction (PCR) strategy and degenerated oligonucleotides from conserved sequences of the *mariner* central region. The presence of different subfamilies in the same species, together with the description of closely related *mariner* elements in distant species, suggests that the phylogenetic distribution of *mariner* in insects was due both to horizontal transfer and to an early divergence with stochastic loss in some branches^{3,4}. Robertson failed, however, to amplify any sequence in 18 vertebrate-representative species, and he presented no data on the presence of *mariner* elements in lower invertebrates.

While analysing the 5' region of the planarian *Dugesia* (Girardia) *tigrina* (Platyhelminthes) homeobox gene *Dth-2* (ref. 5), we found an incomplete open reading frame (ORF) which bore a remarkable similarity to the transposase coded by the *Drosophila mariner* element⁶. We then isolated a genomic DNA fragment which included a complete transposon referred to as planarian *mariner-1* owing to its high similarity to the *mariner* transposon structure (see figure). It contains a single ORF of 339 amino acids, which shows, respectively, 35 and 55 per cent similarity to the Mos-1 *D. mauritiana* ORF (ref. 7) and the putative ORF of *Hyalophora cecropia mariner*-like element³. It also shows a significant similarity (31 per cent) to a recently available sequence of *Caenorhabditis elegans* element (GenBank accession number M98552).

This *mariner* element is present in about

8,000 copies in the *D. tigrina* genome and it is very conserved at the restriction level, as determined by genomic Southern analysis (data not shown). This high copy number, the presence of an uninterrupted ORF whose codon bias resembles that of other planarian genes⁸ and the existence of most standard signals of active genes, all point to an active role of the *mariner* element in planarians.

Kidwell points out in News and Views⁹ that *mariner* probably arose before the diversification of insects. Nevertheless, inter-specific horizontal transfer cannot be ruled out as it may explain *mariner* sequence similarities between some distantly related insect species. In planarians, both arguments are reinforced: we have not detected *mariner*-like sequences in the genome of the planarian *Dugesia* (Schmidtea) *mediterranea*, which suggests either horizontal transfer to *D. tigrina* or loss of the *mariner* element in *D. mediterranea* lineage. The former hypothesis may imply that horizontal transfer could involve not only species of the same class, but also species of different phyla: the latter, that *mariner* ought to be considered ancient as it may have existed before the radiation of Platyhelminthes from the branch leading to higher metazoans¹⁰.

The wide host distribution in insects and the ability of integration in another *Drosophila* species¹¹ have suggested the *mariner* element as a vector for the stable transformation in arthropods^{1,3,9}. The presence of apparently active *mariner* transposons in planarians opens a novel perspective as these elements could be developed as an integrative system for a wide range of animal species or phyla.

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ROBERTSON REPLIES — The *mariner* discovered by Garcia-Fernández *et al.* in *D. tigrina* is remarkably similar to those of insects. Indeed, it clusters phylogenetically within the cecropia subfamily of insect *mariners*, having 75 per cent amino acid identity with certain ant elements¹. This similarity must result from horizontal transfer from an insect lineage. Other *mariners* have recently been found beyond insects. Within arthropods, they occur in a centipede and an ascaid mite¹, and A. Jeyaprakash, M. A. Hoy and I have obtained *mariner* sequences from a phytoseiid mite. These elements cluster within the mauritiana subfamily of insect *mariners*¹. P. Capy (personal communication) has detected *mariner* elements in two isopod crustaceans.

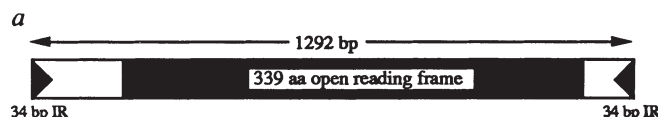
Beyond arthropods, two distinct *mariners* have been found in the nematode *C. elegans*. There are two copies of the element mentioned by Garcia-Fernández *et al.* in GenBank, in separate cosmid

- Robertson, H. M. *Nature* **362**, 241–245 (1993).
- Hartl, D. L. in *Mobile DNA* (eds Berg, D. E. & Howe, M. M.) 5531–5536 (Am. Soc. Microbiol., Washington, DC, 1989).
- Lidholm, D.-A., Gudmundsson, G. H. & Boman, H. G. *J. biol. Chem.* **266**, 11518–11521 (1991).
- Maruyama, K. & Hartl, D. L. *J. molec. Evol.* **33**, 514–524 (1991).
- Garcia-Fernández, J., Baguña, J. & Saló, E. *Development* **118**, 241–253 (1993).
- Jacobson, J. W., Medhora, M. M. & Hartl, D. L. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8684–8688 (1986).
- Medhora, M., Maruyama, K. & Hartl, D. L. *Genetics* **128**, 311–318 (1991).
- Garcia-Fernández, J., Baguña, J. & Saló, E. *Proc. natn. Acad. Sci. U.S.A.* **88**, 7338–7342 (1991).
- Kidwell, M. G. *Nature* **362**, 202 (1993).
- Riutort, M., Field, K. G., Turbeville, J. M., Raff, R. A. & Baguña, J. *Can. J. Zool.* **70**, 1425–1439 (1992).
- Garza, D., Medhora, M., Koga, A. & Hartl, D. L. *Genetics* **128**, 303–310 (1991).

a, Schematic structure of the *Dugesia tigrina mariner-1* element. Black box, ORF encoding the presumptive transposase; triangles, terminal short inverted repeats. b, Comparison of the planarian *mariner-1* ORF with the transposase of the active *D. mauritiana* Mos-1 *mariner*, and the reconstructed ORFs of the *H. cecropia mariner*-like element (MLE) and of the *C. elegans* element. Dashes, identical residues (gaps have been introduced for optimal alignment); asterisks, stop codons in the aligned reading frames; hash sign, positions where a frameshift has to be considered to maintain the alignment.

b

Planarian <i>mariner-1</i>	MEISEIRILMKYEFHGRATTQAVGNINSVYPTQAVTQTVAHWFKRFRSGDFMSNQPSRPEIKVDNALKADVEADSSQSALELASKFGVAKSIILIHKLQINKVKKLKDW
<i>H. cecropia</i> MLE	-ANMKY-YIYE---Y-TSAAETARR-N-GAG-AKESK-RF-Q-----I-LQ-----G-----E-EI-#-*-P-TS-I-AG---SDKT-V-Y-----E*-
<i>D. mauritiana</i> Mos-1	MSSFVPNKQT-TVLIFC-LKK-AAESHRMLVEAFGE-VP-VR-CER--Q-K-----VDDKEHGK-PKRYEDAE-Q-LLDE-DA-TQKQ-EQLE-SQQAQVSNR-REMG-IQ-VGR-
<i>C. elegans</i>	MTENLLA-RHAL-GVFL---PQSCNCNE-RR-MCV-LGKSS--YN-MKF--EK-TKKNY-LDDK--*DRSLRDLDEIDRAL-D-RATSR--SATLKHPQR--INH-QKTG-IE-FGQL
121	
Planarian <i>mariner-1</i>	VPHELKDEHKQRLDACLSLLSRNKADPFLHRIVTCDEKWMYIMDNKRSSQWLDPEPPKHKCRKVKHQKLMVTWVWSSYGVHIDFVMPGTSITSDVYCSQLDDMMKLAIKQPKMFN
<i>H. cecropia</i> MLE	---SESNL-T-V-CYVT--N-HNNE.....K-----G-L--N-GD-A-S-----LT---L-VF--T-A-----S-LKC-QT--V-I-YQ--QA-K-E--A-H-RLV-
<i>D. mauritiana</i> Mos-1	---NERQMER-KNT-EI---Y-RKS-----G---FFNSP--KKSIV--GQ-ATSTARNRFG-T-LC---DQS---Y-ELLK--ETVNTAR-QQ--INLNRA-QR-R-EYQK
<i>C. elegans</i>	---K-S-SQ-NCVF#LS-S-T-KRTTDWVKD-I-GND---VL-VSHT-KKE-VPVE-TATPDL-EL-G--VLLSIGRD-K--SRELLPDFAT-NAGL--I*-EKVVAHRLHR--...
241	
Planarian <i>mariner-1</i>	R-LTPILLHDNARPHSAKNTVAKLQQLGLETLRHPPYSPDLAPTDFHFFQSLDNFLSGKNFTSSGAVKTAQFQFIDSRSESVFYTKGLNVLPKQWQCVQDNMGYFD
<i>H. cecropia</i> MLE	-.SRSL-----T-Q-TT--NK-Q-C-----I-----RN-----H-K-N-YSV-Q--K---R-PHA-FN--I-E--VR--K-IN-N-A--
<i>D. mauritiana</i> Mos-1	-QHRV-F-----PS-T-RAVRDT-ET-NW-V-P-AA-----S--L-A-MGHA-AEQR-D-YES--KWLQ-WFAAKDDE--WR-IHK--ER-EK--ASD-K-E-
<i>C. elegans</i>	-GSKLL-----TTFK-RQ-----TV-IQI-SY-S--G-----L-R-Q-H-A-QK-HDRKV-E-GLDD-FA-*SQE-*AE-TVQ---C-EVIGIN-K-ITH



(ZK370 and C30A5) compiled by the Nematode Genome Sequencing Project². We have also published an alignment of this transposase with those of the insect elements³. In addition, M. Sedensky and P. Morgan (manuscript in preparation) have found a quite distinct *mariner* in *C. elegans*. These two *mariners* do not cluster with insect *mariners* and represent additional subfamilies. Finally, T. Langin, P. Capy and M. J. Daboussi (manuscript in preparation) have found *mariner* elements in a fungus. These

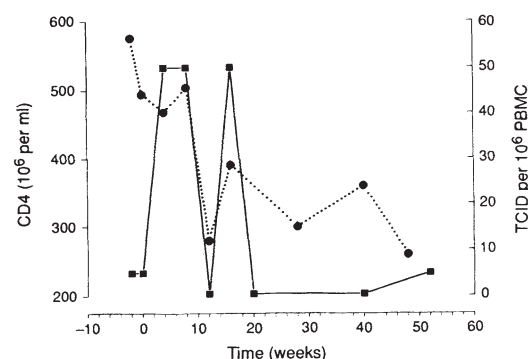
parasitic genes are therefore of great antiquity, and have infiltrated the genomes of many organisms.

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1. Robertson, H. M. & MacLeod, E. G. *Insect molec. Biol.* (in the press).
2. Sulston, J. et al. *Nature* **356**, 37–41 (1992).
3. Robertson, H. M., Lampe, D. J. & MacLeod, E. G. *Nucleic Acids Res.* **20**, 6409 (1992).

HIV quiet but not silent

SIR — Much attention has been focused on the site and quantification of HIV infection in the early stages, before significant immunosuppression. It has been implied that asymptomatic HIV infection is



Fluctuations in infectious viral load in PBMC during cells, asymptomatic HIV infection. Circles, CD4; squares, tissue-culture infectious dose (TCID).

clinically silent, and that viral replication may be restricted largely to the lymph nodes^{1,2}.

Although early HIV infection is often described as asymptomatic, it is far from silent clinically or virologically. In practice, all HIV-positive people have some degree of fluctuating lymphadenopathy throughout the asymptomatic stage, which may or may not be sufficiently pronounced to be termed persistent generalized lymphadenopathy³. Hypergammaglobulinaemia is also detectable early in the course of HIV infection, and thrombocytopaenia may occur throughout this period. Even when the CD4 count exceeds $500 \times 10^6 \text{ l}^{-1}$, HIV-positive people may experience recurrent folliculitis, especially in the beard area, seborrhoeic dermatitis on the face and chest and generalized xeroderma; pityriasis versicolor infection is common.

Although these phenomena can be thought of as minor opportunist infections, "asymptomatic" subjects also report bouts of self-limiting night sweats, generally without objective fever, but often associated with profound lethargy and fatigue⁴.

These episodes may be temporally related to the peaks of infectious virus load seen in the peripheral blood lymphocytes. Although viral burden may be greatest in lymph nodes early in the course of HIV infection, infectious virus can be recovered from peripheral blood mononuclear cells (PBMC) in most HIV-positive patients throughout the course of infection, from primary infection onwards⁵. The level of infectious virus load in PBMCs fluctuates considerably over time in asymptomatic subjects (see figure), although the full significance of these fluctuations is still unclear.

HIV leads to systemic manifestations throughout the course of infection, and although these conditions may not be life-threatening, they are far from silent.

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Mouse coat colour reconsidered

SIR — Jackson's interesting discussion¹ in News and Views of coat-colour genes proposes an unnecessarily narrow hypothesis and ignores a more likely one. Jackson depicts the agouti protein as a competitive antagonist that binds to the melanocyte-stimulating hormone receptor (MSH-R), but fails to consider an alternative possibility, that the agouti protein acts instead as an agonist on a separate receptor, triggering a pathway that opposes downstream effects of the MSH-R. Ignoring the alternative hy-

pothesis runs the risk of overlooking a potentially important determinant of coat colour and inadequately explains the pleiotropic effects of a dominant mutation at the agouti locus.

The MSH-R and the agouti locus control coat colour via opposing effects on a bifurcated biochemical pathway that converts tyrosine to either pheomelanin (yellow coat) or eumelanin (black coat). Activation of the MSH-R promotes synthesis of eumelanin², while the agouti protein enhances production of pheomelanin³ (the default pathway). The agouti protein could act by binding directly to the MSH-R, as Jackson suggests. Alternatively, a distinct receptor for the agouti protein could activate a signalling pathway that independently regulates melanin production in the target cell. Push-pull regulation by opposing hormones is common in nature (for example heart rate, glycogenolysis). The notion of a naturally occurring, physiologically relevant competitive antagonist acting on a cell surface receptor is intriguing, but to our knowledge, without precedent.

Ubiquitous overproduction of the agouti protein in mice with a dominant yellow mutation at the agouti locus causes obesity, diabetes and tumours in addition to a yellow coat colour³. Injection of MSH can override the effect of this mutation on coat colour⁴ but has not been shown to counteract the other effects. To explain this Jackson broadens the hypothetical antagonist activity of the agouti protein to block other (non-MSH) receptors¹. A simpler model would allow the agouti protein to induce obesity, diabetes and tumours by activating its own receptor.

If the agouti protein has a distinct receptor, why has its gene eluded coat-colour geneticists? It is well known that genes that are necessary for viability, or that encode proteins with redundant functions, can be refractory to traditional linkage analysis. This is probably why the genes for adenylylcyclase and $G_{\alpha s}$ are not defined by coat-colour loci although the protein products of both genes are undoubtedly necessary for MSH-driven coat darkening.

Studies with purified agouti protein will eventually show whether it is a competitive antagonist or acts on its own receptor. Until then it seems prudent to consider all the options.

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1. Pantaleo, G. et al. *Nature* **362**, 355–358 (1993).
2. Embretson, J. et al. *Nature* **362**, 359–362 (1993).
3. Centers for Disease Control Morbid. Mortal. Wkly Rep. **31**, 248–251 (1982).
4. Weber, J. & Pinching, A. J. in *The Management of AIDS Patients* (eds. Miller, D., Weber, J. & Green, J.) 1–35 (Macmillan, London, 1986).
5. Bieniasz, P. et al. *AIDS* **7**, 803–807 (1993).

1. Jackson, I. J. *Nature* **362**, 587–588 (1993).
2. Robbins, L. S. et al. *Cell* **72**, 827–834 (1993).
3. Bultman, S. J., Michaud, E. J. & Woychik, R. P. *Cell* **71**, 1195–1204 (1992).
4. Jackson, I. J. *BioEssays* **13**, 439–446 (1991).

The Bambi syndrome

Adam Kuper

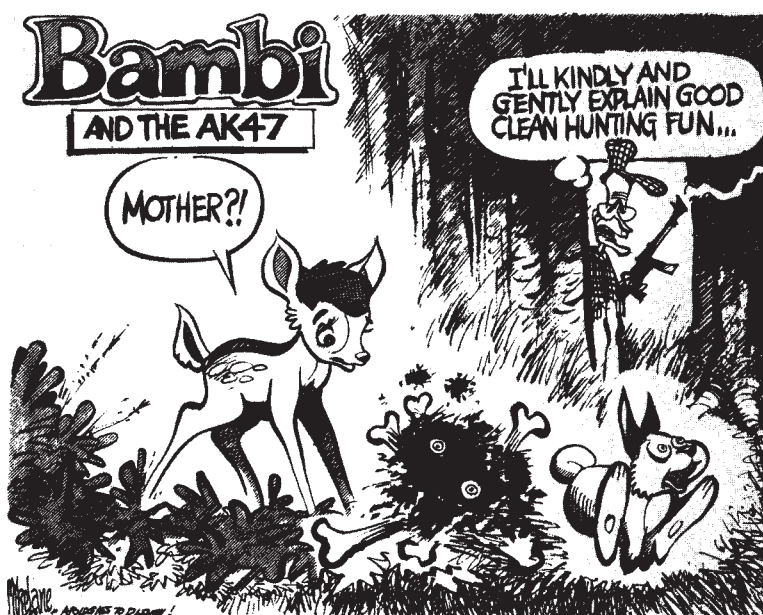
A View to a Death in the Morning: Hunting and Nature Through History. By Matt Cartmill. *Harvard University Press: 1993. Pp. 331. \$29.95, £23.95.*

In 1925, Raymond Dart described the first fossil *Australopithecus*, the ape-like hominid now known to have lived in southern and eastern Africa from about 4 to 1.4 million years ago. As specimens accumulated in the decades that followed, it became clear that the creature was predominantly bipedal — no surprises there. What caused consternation, however, was the fact that the creature's brain was astonishingly small, little different from that of a chimpanzee. This came as a shock to darwinians, who, since Huxley, had argued that human evolution was paced by growth in cranial capacity and brain power. It was a long time before the likes of Sir Arthur Keith would accept that *Australopithecus* was a hominid at all, rather than an extinct species of ape.

But if the first hominids were not, after all, distinguished by their capacity for reason, what did mark them off from other primates? The specimens of *Australopithecus* that Dart and Robert Broom turned up in the following decades were associated with large deposits of bones of wildebeest and buck, and Dart concluded that one great difference between these creatures and the predominantly vegetarian apes was their taste for meat. This led them to make bone tools, mainly for hunting and butchering their kills. Indeed, Dart took the view that *Australopithecus* was such a ferocious hunter that he killed and ate his fellow creatures. Hunting, warfare, meat-eating, perhaps even cannibalism, made us human. Other anthropologists took the argument a step further. The demands made by successful hunting — planning, scouting, investment, cooperation between men and a division of labour between men and women — were at the root of the characteristic human forms of social organization, including the family itself.

This model of human evolution became the orthodoxy in the 1960s but it is now in tatters. It turns out that other primates, notably chimpanzees, do hunt, and that

Australopithecus itself was not a very successful hunter and did not make tools. Indeed, the creature was more likely to be the victim of other predators, its remains often found in caves associated with the bones of game animals because predators and scavengers dragged their prey to these shelters. There are now also doubts about the hunting abilities of *Homo erectus* and even of early *Homo sapiens*, including the



Bambi — symbol of wilderness and the natural order. In this cartoon from the *Baltimore Evening Sun*, Bambi's mother is killed by President George Bush. It appeared during the furore in 1989 over the US importation and sale of semiautomatic rifles.

Neanderthals.

So why did Dart's theory persuade so many scientists? Matt Cartmill suggests that it seemed to offer a key to what had happened in Europe in the first half of the twentieth century. But this particular hunting hypothesis is only the latest in a series of myths that represent us as hunters by nature. After disposing of Dart, Cartmill traces a long line of ideas about hunting in the Western tradition (which he represents in a rather old-fashioned way as stemming from two cultures, Ancient Judaism and Classical Greece and Rome).

The Garden of Eden was a vegetarian paradise. Noah was given dispensation to eat the flesh of animals, but the Jewish tradition imposed complicated taboos on hunting and on the consumption of meat. The Greeks, however, made a cult of hunting, and two of their gods represented images of the relationship between

ourselves and the beasts. The virgin huntress Artemis — the Roman Diana — is at once huntress and guardian of the animals, a sort of divine game warden. Dionysus is part of the wild world, the hunter as beast of prey, free from the restraints of civilization. The Romans took up the Greek cult of hunting, making it a circus spectacle; one, however, that might provoke pity, as when the crowd rose up in protest against the slaughter of African elephants, to the surprise of Pompey, who had arranged the spectacle. "The whole affair was attended by a sort of pity," Cicero wrote to a friend, "and a feeling that these huge animals have something in common with humankind."

The early Christians demonized wild nature, reading the Genesis myth as a charter for human control of nature, but Cartmill argues that as the frontiers of the forest were pushed back in mediaeval Europe, an Edenic image of sacred nature began to spread. At the same time, the forest was increasingly controlled by the aristocracy, and hunting — the analogue to war — became a defining privilege of the nobility, the peasantry being reduced to poaching.

Since Cicero, and increasingly since the Renaissance, a number of texts also denounce hunting, for secular, humanist reasons, as cruel, foolish and uncultured. The Romantics were inclined to rate nature above culture, and in some cases preferred wild beasts to humans. Nevertheless, the criticism of hunting did not make much headway against the general belief that humans are naturally carnivorous and predatory.

Moreover, darwinian theory was widely perceived as a blow for the hunting lobby (and, indeed, for the war party). A man such as Theodore Roosevelt saw himself as the darwinian hunter, emperor over man and beast. Cartmill contrasts him interestingly with the democratic, innocent frontiersman, in touch with nature — Davy Crockett (or even Burroughs's Tarzan, perhaps), nature's sheriff rather than an imperialist commissar, an American Artemis to Roosevelt's Dionysus.

But the darwinian and the Romantic myths have grown together in contemporary America. The environmental movement draws on darwinian ecology and on a Romantic — perhaps Christian — reverence for nature. Cartmill seems to think

From A View to a Death in the Morning

that myth has at last caught up with true science. Yet in perhaps his most original chapter he traces the story of Walt Disney's Bambi, the innocent faun, regarded by the hunters' lobby in the United States as its most potent challenge. If this is the popular myth behind conservationism, then the American nature lobby may have more in common with the readers of Aesop than with darwinians.

Like Keith Thomas's *Man and the Natural World*, which it complements in many ways, this book is an elegant, erudite, stimulating essay on the history of Western ideas about humans and nature; and if it is a story with a moral, then the moral is more appealing than Dart's. But Cart-

mill's skill as a writer cannot disguise the fact that this is rather old-fashioned intellectual history. Although he does sketch in the local, temporary ecology of some debates, the overall impression is of an evolutionary tree of hunting myths, or a fossil series, each specimen pregnant with its successor. Seduced by this genealogical fallacy, he makes no use of the anthropologist's cross-cultural casebook. This is a pity, since all these hunting myths are examples of the universal practice of totemism. □

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should be taught by exposing students to groups of words in the same category, for example, ten words describing people such as 'accomplice', 'virtuoso' and 'novice'. After the words are learned the teacher gives a clue to one of them — for instance, 'crook'. The students shout 'accomplice', and are asked to explain the connection between the two words. The object is to build up schemata that incorporate sets of concepts and their inter-relationships. Sensibly, Bruer points out that many elementary reading texts require a background of concepts that the students simply do not have. It is no use giving them a passage on the American Revolution that refers to 'the 13 colonies' if they have no idea what that means. This problem can be solved either by changing the texts or by giving children more background information before they read them.

Bruer claims that these innovations in teaching, most of which were inaugurated and tested by psychologists, stem from advances in cognitive psychology. Perhaps they were triggered by that discipline, but they come perilously close to ingenious common sense. Nevertheless, they all appear to work and when compared with conventional teaching they raise students' ranks in tests by anything between 20 and 40 percentile points.

There are, however, problems. All the methods are time consuming, both for the teacher and for the pupil. Using the techniques for teaching physics already described, students took more than a week to learn Newton's laws instead of the normal day or two. Perhaps conventional methods would do just as well if as much time were devoted to a given topic. It is, moreover, well known that introducing any novelty into an institution produces good results — for a time: both the teachers and the pupils are likely to be more enthusiastic and better motivated.

Nevertheless, Bruer is on the side of the angels, in that he believes students should be taught to understand and to think, but the conservatism of school teachers and the ignorance of governments are likely to die hard. Answering multiple-choice questions requires neither originality nor thought, yet many of the new tests in British schools will be based on them. Most American universities use such tests as a method of assessment and to their shame some British universities have now introduced them. Writing an essay or solving a problem requires thought and some originality, but few now are interested in those capacities. The current ethos is that the teacher is there to cram pupils with facts, if only to impress examination boards and quiz masters. □

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How our teachers ought to teach

Stuart Sutherland

Schools for Thought: A Science of Learning in the Classroom. By John T. Bruer. MIT Press: 1993. Pp. 324. \$29.95, £26.95.

IN recent years a mindless craze has swept the United States and Britain. Trivial Pursuit has ruined many an American dinner party and the quiz is wrecking the atmosphere of the British pub. Perhaps the popularity of the quiz arises from the rote learning so widely practised in schools throughout the world. I was solemnly taught arithmetic operations — borrow one in subtraction and so on — without being given any idea why these procedures worked and indeed without any clear understanding that numbers were made up of units, tens, hundreds and so on. Nor were the solutions to algebraic equations or those of logarithms any better explained. One simply had to work it out for oneself.

Schools for Thought suggests ways of overcoming these problems. First, it argues that in executing procedures children often suffer from systematic misconceptions, which make them repeat the same error again and again. It is no use putting a cross against a subtraction wrongly performed; the nature of the error should be identified and the correct procedure explained. Children may consistently forget to borrow one, they may fail to carry a borrow across two places and more bizarrely they may always subtract within a column the smaller number even if it belongs to the subtrahend. Such incorrect procedures are hard for the teacher to detect as a single error may be random rather than an example of the use of a wrong rule: they can, however, readily be detected by computer if the child's efforts are fed directly to it.

J. T. Bruer argues that new concepts will be most readily grasped if they are

linked to what the pupil already knows. Most children starting school can already count a group of objects by touching each in turn and uttering the next number. To facilitate their grasp of arithmetical procedures, they can be presented with differently sized blocks representing units, tens and hundreds. The blocks correspond to the figures in a sum, which the children do in the normal way while simultaneously manipulating the blocks: this technique, which has been in use for some time, bears more than a slight resemblance to the old-fashioned abacus.

Bruer maintains that students learn best through disputation with one another and the teacher. One outstanding American teacher of physics found that his pupils, who had done excellently in examinations, could not transfer their formal learning to "the physics of everyday situations, and they showed little understanding of basic physical concepts such as force, motion and gravity". Equations can be learnt by rote, but that does not constitute understanding. To overcome this, he probed his students to discover their naive opinions on physical laws. Opinions varied — some might think that an object in a vacuum will be weightless, whereas others believe it will have weight. The disagreement is resolved by demonstrating experimentally that bodies do have weight even in a vacuum. Again, most people bring to physics such false beliefs as that only a moving object can impart a force and few understand the effects of a force applied to a moving object, but pupils can apparently readily grasp the correct laws by experimenting with a computer display programmed to simulate Newton's laws. They learn by subjecting a moving sphere to different forces and observing the results.

On the use of new methods of teaching reading and writing, Bruer is less persuasive. He suggests that vocabulary

Our genetic legacy

Christopher Wills

The Language of the Genes: Biology, History and the Evolutionary Future. By Steve Jones. HarperCollins: 1993. Pp. 251. £16.99. To be published in the United States later this year by Doubleday.

THIS book is an expansion of the Reith lectures that the geneticist Steve Jones gave on British Broadcasting Corporation radio in 1991. It was with some bemusement that I realized that substantial numbers of people in England still sit down and listen, for tens of minutes at a time, while somebody intelligent and witty talks about something complicated and interesting on the radio. No commercials destroy the speaker's flow of thought, and he does not have to field telephone calls from people who in the course of the programme have found martians under their beds. What heaven for somebody with the academic's urge to inform without being interrupted.

Jones rises to the occasion. The book is urbane and casts its net widely, covering topics from the probable genetic evolution of our species through the complexities of human sociobiology to the promises and perils of genetic engineering. He says many eminently sensible things along the way, and I am sure that the listeners to his lectures ended up with a broad appreciation of the complexities of human genetics and evolution.

The two form, as Jones makes plain, a seamless whole. Our genome is enormously complex, and our understanding of how it operates to produce adult humans is growing apace. Only a part of this understanding will come from the Human Genome Project, for our genes cannot be comprehended except in their evolutionary context. Jones exhibits the biologist's distrust of Big Science when he bemoans the shift of research funds into "... Programs, Institutes and Centers [that] give the predictable new life at the expense of the unexpected". And indeed much of the book, except when it briefly discusses such megaprojects as the search for the cystic fibrosis gene, is a celebration of the thousands of cases in which small science has illuminated some fascinating aspect of how we work and how we have evolved. And often he peeks into areas that cry out for a more thorough exploration. One that particularly caught my attention was the old and unpleasant eugenic idea of anticipation, in which eugenicists bemoaned the fact that genetic defects such as myotonic dystrophy seemed to get worse as one generation succeeded the next. It now turns out that our understanding of a number of diseases

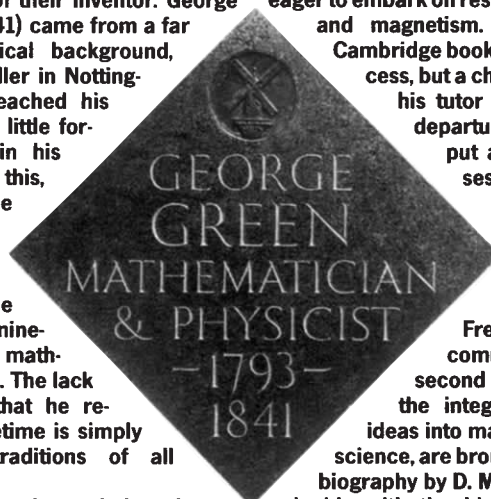
The Green movement

GREEN'S functions, to many young mathematics students, seem to be plucked out of thin air to solve second-order differential equations. The same could almost be said of their inventor. George Green (1793–1841) came from a far from mathematical background, working as a miller in Nottingham until he reached his thirties, and had little formal education in his youth. Despite this, his formidable mathematical achievements made him one of the pioneers of nineteenth century mathematical physics. The lack of recognition that he received in his lifetime is simply in the best traditions of all pioneers.

The Green's function technique formulated in his first and most important publication, the *Essay on the Application of Mathematical Analysis to the Theories of Electricity and Magnetism*, has become a staple of classical physics and quantum mechanical calculations. Nuclear physics, quantum electrodynamics and radar all owe something to Green. And Green's Theorem, which transforms tricky problems of summation over volume to (one hopes) more tractable problems in terms of area, is familiar to most physicists. But had it not been for the interest of the young William Thomson, later Lord Kelvin, the work might have been lost. Thomson

came across a reference to "the ingenious Essay by Mr Green of Nottingham" in 1845, when he had just reached the end of his undergraduate career and was eager to embark on research in electricity and magnetism. He scoured the Cambridge bookshops without success, but a chance meeting with his tutor on the eve of his departure for Paris finally put a copy in his possession.

The rest, as they say, is history. The subsequent stir caused among the French mathematical community, the essay's second publication and the integration of Green's ideas into mainstream physical science, are brought to life in a new biography by D. M. Cannell, timed to coincide with the bicentenary (*George Green, Mathematician and Physicist 1793–1841: The Background to his Life and Work*; Athlone, London, £35, \$70). Celebrations for the bicentenary on 14 July may lift Green from his present obscurity. Next week, in addition to lectures at Nottingham University and the Royal Society in London, a memorial (above) will be dedicated to Green in Westminster Abbey, as will a stained glass window in Gonville and Caius College, Cambridge, where Green began a belated mathematics degree at the age of 40, five years after publishing the *Essay*. L.M.



— fragile X syndrome, myotonic dystrophy, spinobulbar muscular atrophy and most recently Huntington's disease — has provided a molecular explanation for anticipation. The eugenicists, repellent though their conclusions might have been, were at least right about the phenomenon. This subject alone deserves a whole book.

In such a wide-ranging book there are inevitably some places where there is room for disagreement. Jones says: "A chimp may share ninety-eight per cent of its genes with a typical human being but it is certainly not ninety-eight per cent human: it is not human at all — it is a chimp". Actually, a chimpanzee shares essentially 100 per cent of its genes with a human — it is small details of many of those genes that are different. And there are far more similarities between chimps and humans than there are differences — we do, after all, have 99.8 per cent of our evolutionary history in common. I also think that Jones rather slights the contribution of palaeontologists to our understanding of human evolution, though he is clever in doing so: "If geography is about maps and biogra-

phy about chaps, human paleontology is mainly about gaps". While palaeontologists have sometimes been overenthusiastic in interpreting their remarkable discoveries, they have still told us far more about our prehistory than all the molecular family trees in our genomes could ever do.

Finally, Jones suggests that because of the recent relaxation of selective pressures on at least part of our species, "we are as near to our biological Utopia as we are ever likely to get". I hope we are, but I doubt it. As the popular T-shirt says, "Malthus was right!". We cannot multiply indefinitely and savage the planet as we are doing without the evolutionary chickens eventually coming home to roost. Still, this intelligent and informative book deserves to be read widely, since it provides a vivid demonstration of what a remarkable and fragile product of evolution our species is. □

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Secret history

Nicholas Kurti

Operation Epsilon: The Farm Hall Transcripts. Institute of Physics Publishing: 1993. Pp. 313. £14.95. US distributor, University of California Press.

ON 3 July 1945, ten prominent German scientists involved in the German wartime Atomic Energy Project arrived at Farm Hall, a country house near Cambridge in England, where they remained for 6 months. Their whereabouts were kept secret, their communications with their families were sporadic and slow and they were kept in darkness about the reasons for their internment.

Their conversations, picked up by microphones, were continuously monitored. Anything nontrivial was recorded on shellac-covered reusable disks and most of these recordings were transcribed by the 'listeners'. The two officers in charge (Major T. H. Rittner and Captain P. L. C. Brodie) analysed the transcripts, had part of them translated and summarized the rest. These translations, summaries and the officers' comments on the operation, code-named Epsilon, formed the Farm Hall reports, numbered FH1–FH23/24 and classified TOP SECRET.

Their existence was first surmised when Samuel Goudsmit, the leader of the American Scientific Intelligence Mission ALSOS, reported in 1947 in his book the reactions of the German scientists on hearing the news of the Hiroshima bomb. First, they did not believe that it was an atomic bomb; then, seeing their faith in German scientific superiority shattered, they were dejected and in despair. But, according to Goudsmit, they then decided to stress the fact that they had been working only on nuclear reactors and that the community of German scientists would never have consented to work on the bomb. It is ironic that Goudsmit's suggestion, now known to be ill-founded, of a plot to explain away the nonexistence of a German bomb, should have become years later, in the writings of R. Jungk, L. Sciascia, W. Kaempfert and T. Powers, the legend that moral scruples prevented German scientists from developing the bomb*. Thus, comparing the attitudes of the Allied and the German scientists, Sciascia wrote that the Germans "were worried by the bomb, afraid, anguished, while the Allies conceived it, studied it, got it ready for use without hesitating, indeed almost lightheartedly and handed it over . . . to the politicians and war-mongers". To the best of my knowledge, this legend was never explicitly disowned by the German scientists. To get to the

truth there were many attempts by scientists and historians to have the Farm Hall documents de-classified, but the answer was always a firm 'no', with a reminder that the Lord Chancellor (Lord Mackay), the ultimate authority in this matter, was under no obligation to give the reason for his decision.

It was then thought that, where all else had failed, a direct approach to the Lord Chancellor might succeed. A letter signed by 17 scientists and historians headed by the presidents of the Royal Society and of the British Academy was accordingly despatched on 23 December 1991 to the Lord Chancellor who, on 13 February 1992, informed the president of the Royal Society that the Farm Hall documents would be released to the Public Record Office on 14 February.

These documents are now available in book form, thanks to the laudable enterprise of Institute of Physics Publishing. It is a handsome volume with an excellent introduction by Sir Charles Frank that puts uninformed readers in the picture and helps them to get the gist of the often confused conversations.

As to the absence of work on the bomb in Germany, Frank suggests that this may have been due to the gross *overestimate* by Heisenberg of the critical mass for an explosion in ^{235}U and he believes that the Allies would have reached the same conclusion but for the fact that in March 1940 Otto Frisch and Rudolph Peierls seriously *underestimated* the critical mass. There is a fundamental difference between the two approaches. Frisch and Peierls, using for fission cross-section a value *estimated* from theory, *calculated* the critical mass to be 600 g. Even a four-times smaller value for the cross-section would have given a bomb of only about 30–40 kg, nothing like the 500 or 5,000 kg mentioned (p. 73) five years later by Heisenberg, who admits that "we don't know the order of magnitude. We can assume that they [the Allies] have some method of separating isotopes of which we have no idea." It is significant that the gaseous diffusion separation plant proposed by F. E. Simon (December 1940) was designed to produce enough ^{235}U for a dozen 30-kg bombs a year. In brief, the Frisch–Peierls memorandum together with the Simon report gave a sober assessment of the feasibility of an atomic bomb, and to my knowledge there is nothing in contemporary German archives approaching the scientific and technical contents of those reports.

In his "Archival Note", Frank gives interesting details about the two sets of Farm Hall reports, one in the UK Public Record Office and the other in the American National Archives. The United States declassified the Farm Hall reports in August 1961 but, because of the UK embargo, they could not be made available under the Freedom of Information Act.

What fraction of the words spoken at Farm Hall are reprinted verbatim in the reports? On the evening of 6 August, after the news about the Hiroshima bomb, the conversations went on for seven-and-a-half hours, but only about 50 minutes (10 per cent) is reproduced, the rest is summarized. For the whole six months of Operation Epsilon, there are only four hours of verbatim reporting. Moreover, as is now officially admitted, the transcripts from the original recordings — in other words the prime historical material — no longer exist. This is worrying and one can but hope that historians and archivists will try to find out whether this was an isolated case of carelessness, an act of stealthy censorship or the result of an attitude that would regard material in a foreign language as disposable once it had been translated into English.

Although the title of the book is, as Frank points out, a misnomer, this in no way detracts from the volume's value. It sheds light both on the 'guests' and on their 'hosts'. It is fascinating to observe the varied and ever-changing moods and attitudes of the scientists: truculence and good-humoured resignation, complaints and compliments, despair and hope alternate and merge. Moral scruples about working on the atomic bomb are not evident. Equally interesting is the attitude of the 'hosts': the two officers did their best for their guests but without much help from above. It seems that Operation Epsilon had no clear purpose and was organized impromptu. An example: the scientists were not freed until 3 January 1946, therefore preventing them from spending Christmas with their families.

This book will remain for many years a happy hunting ground for historians, political scientists and psychologists. □

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New in paperback

Consciousness Explained by Daniel C. Dennett. Penguin, £7.99. For a review, see *Nature* **356**, 26 (1992).

Left Handers by Stanley Coren. John Murray, £9.99. See *Nature* **356**, 637 (1992).

The Computer from Pascal to von Neumann by Herman H. Goldstine. Princeton University Press, \$23.50, £13. See *Nature* **243**, 307 (1973).

The Fall-Safe Society: Community Defiance and the End of American Technological Optimism by Charles Piller. University of California Press, \$12. See *Nature* **355**, 124 (1992).

The Formation of Science in Japan by James R. Bartholomew. Yale University Press, \$23, £14.50. See *Nature* **343**, 225 (1990).

*See, for example, *Nature* **359**, 473 (1992), **362**, 117 (1993) and **363**, 311 (1993).

Seismological detection of a mantle plume?

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A detailed seismological study of the mantle beneath the Bowie hotspot, west of Canada, reveals a zone of low seismic velocities. The anomaly, probed at a depth of ~ 700 km, adds a delay of 0.15 s to the travel times of seismic waves traversing a region ~ 150 km in diameter, located ~ 150 km away from the vertical projection of the Bowie seamount. This observation suggests that a mantle plume is present in the lower mantle beneath Bowie, with the amplitude of the anomaly implying a temperature contrast of about ~ 300 K.

OVER twenty years ago, Morgan¹ proposed that mantle plumes (diapirs), rising from the core–mantle boundary, were responsible for ‘hotspot’ volcanoes. These volcanoes are seen to form linear chains on the ocean floor, in the middle of tectonic plates², with the most striking example being Hawaii. No better explanation has been proposed since, but several questions remain. Do plumes originate at the core–mantle boundary, or in some hypothetical thermal boundary layer above the 660 km seismic discontinuity³, or at still shallower⁴ depths? Do diapirs rise almost vertically, as the fixity of hotspots with respect to each other suggests, or are they advected by a large convection flow? Is the temperature in the plume 200 K higher than average^{5,6} or 800 K higher⁷? How wide are the plumes, and what initiates them?

Much could be learned if seismological data were to bring insights into the deep structure of plumes. A detailed investigation of the Yellowstone hotspot has revealed low seismic velocities (presumably due to high temperatures) in the lithosphere, down to at least 250 km depth (ref. 8) and possibly 400 km (ref. 9). More recently, evidence for slow hotspot material beneath the lithosphere has emerged from high-resolution global tomography¹⁰. This is consistent with the idea that hot material rising in the plume spreads into a cushion more than 500 km in diameter when impinging on the base of the lithosphere.

What signal is expected at depth?

At greater depths the plume is expected to be only ~ 100 km in diameter^{5–7}. Such a narrow feature would be very difficult to detect. An early indication of a localized slow anomaly at the core–mantle boundary beneath Hawaii¹² was soon discounted as an artefact¹³. Despite the great improvement in regional tomography over the past 10 years^{14–16}, plumes are yet to be detected by seismologists. Hopes are faint, as both the amplitude of the signal and the width over which it occurs are expected to be very small. A lower bound can be constructed on the basis of temperature and diameter derived from petrology and isotope geochemistry^{5,6}, and heat balance^{17,18}. Typical values are a temperature contrast of 200 K and a diameter of 100 km. Assuming that P-wave velocity varies with temperature as $(1/V) dV/dT = -5 \times 10^{-5} \text{ K}^{-1}$ (ref. 19), one predicts an integrated maximum travel-time delay of 0.1 s. To give an idea of how small this is, arrival times reported to the International Seismological Centre (ISC) are rounded to the nearest 0.1 s. This is, however, a lower bound, as the temperature is based on sub-lithospheric processes, and the temperature contrast is expected to increase as one goes

deeper into the mantle. The diameter is also likely to increase with depth, owing to the higher viscosity of the deeper mantle. ‘Realistic’ mantle convection models²⁰ produce diapirs with temperature contrasts of ~ 400 K, and diameters of ~ 250 km, in the upper mantle. This would produce an integrated maximum travel-time delay of 0.5 s.

The delays computed above are simple integrations of velocity anomalies deduced from simple velocity–temperature relationships, and there are several effects that could increase or decrease the P-wave delays: for example, partial melting and anisotropy due to crystallographic alignment in a vertical flow could increase the time delay. Conversely, it has been thought that the time delay could be severely reduced by ‘wavefront healing’, a frequency-dependent wave propagation phenomenon²¹; rays diffracted around the edges of the slow region can arrive before, and obliterate, the Fermat ray, thereby reducing the inferred time delay. However, for the small velocity perturbations and smooth variations expected for plumes, we find this effect to be small. Figure 1 shows synthetic seismograms computed for a plane wave travelling through a uniform region containing a plume-like cylindrical anomaly. Two-dimensional synthetic seismograms are constructed using a parabolic approximation to the full wave equation²². In the propagation plane, the plume-like anomaly is modelled as a gaussian velocity perturbation with a peak value of -2% , and a diameter of 150 km at $1/e$, centred at the origin (0, 0). The wave accumulates a delay of ~ 0.2 s during its passage through the anomaly (Fig. 1a), and this delay remains almost unchanged for distances as great as 1,000 km beyond the plume (Fig. 1b). At 1,500 km, 80% of the maximum time delay persists. We therefore conclude that there are good prospects for detecting plumes in the lower mantle by seismology.

Here we report the results of our attempt to detect a plume beneath the Bowie hotspot. This case was chosen because of its exceptional geometry: rays from 12 earthquakes from the Alaskan subduction zone, recorded at the 120 stations of the Washington Regional Seismic Network (WRSN), illuminate the postulated plume in a fan-like fashion. We determine relative arrival times by multi-channel cross-correlation, with a precision better than 0.05 s. We then separate the different contributions to the travel-time anomalies, and find a slow region, located ~ 150 km away from the vertical of Bowie. We suggest that this anomaly is indeed due to the presence of a plume in the lower mantle.

The exceptional detection geometry

We analyse the first-arriving P-waves from earthquakes at intermediate depth in the Alaskan subduction zone, recorded at the WRSN. Figure 2 shows that rays from these earthquakes to the

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network should travel very close to the expected position of the Bowie plume; if it exists. The Bowie hotspot is thought to be responsible for the Pratt–Welker (or Kodiak–Bowie) linear chain of seamounts, clearly visible on a topographic map, to the northwest of the Bowie seamount^{23,24}. The map is centred on the position of the Bowie seamount.

The advantages of this configuration are as follows. The WRSN is a wide and dense network of more than 120 digital short-period seismographs, which has been in operation for more than 10 years (ref. 25). The network triggers on teleseismic events, and the corresponding seismograms are carefully archived. Intermediate-depth earthquakes are fairly frequent in the Alaskan subduction zone. Because there is both local and regional station coverage, hypocentres are well determined. Rays from these events to WRSN depart from the Alaskan slab almost perpendicular to the strike of the trench, and then travel in a purely oceanic region. The stations are not located too far behind the expected plume, so that we can hope that the small delay we look for is preserved when the waves reach the stations. Finally a detailed study of upper mantle structure beneath the array²⁶ indicates that rays arriving from the northwest are least affected by the presence of the slab that has subducted beneath the continent there.

There are also some disadvantages, related to the Bowie hotspot itself, and to the geometry of the experiment. Although it

has left a clear and typical track of seamounts on the sea floor, the Bowie hotspot is not a 'strong' hotspot (such as Hawaii)¹⁸. There is no marked topographic swell around the Bowie seamount, and alternative positions have been proposed for this hotspot²⁷. As shown in the inset of Fig. 2, the rays that we analyse intersect the hypothetical Bowie plume in the lower mantle, making it impossible to seek the presence of the plume in the upper mantle. The structure beneath WRSN is complex, and strong lateral variations are present, both in the crust and in the upper mantle.

Accurate determination of travel-times

Here we build a profile of travel-time variations as a function of the distance Δ_H from the presumed position of the Bowie hotspot (53.5° N, 135.6° W)²³. Δ_H is measured as the closest distance between each ray and the vertical of the hotspot. As we expect a signal of the order of a few tenths of a second only, emphasis has been laid on getting very accurate measurements. Only intermediate-depth earthquakes with simple, impulsive waveforms have been used (Table 1). The analysis then proceeds in four steps.

First, for each event (e), relative times $t_x^{e,s}$ are determined, using a multi-channel cross-correlation technique²⁵. Figure 3 shows examples of the alignment of the waveforms achieved. We only keep sets of stations (s) yielding an average standard

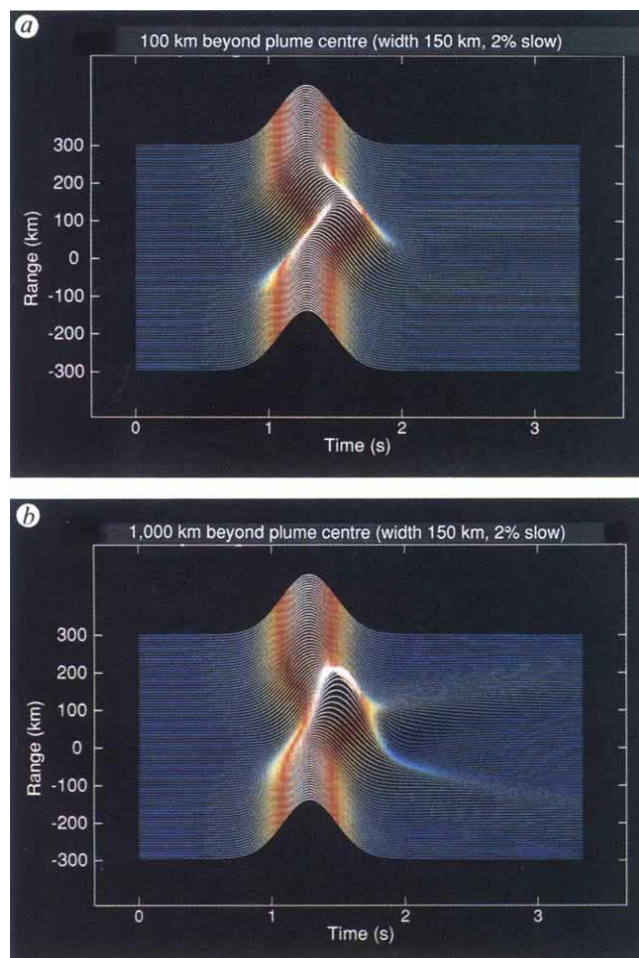


FIG. 1 Synthetic seismograms²² for a plane wave travelling through a cylindrically symmetric plume. a, 100 km; b, 1,000 km beyond the centre of the plume.

TABLE 1 USGS hypocentres of the Alaskan earthquakes used here

Date Year/Month/Day	Time hr:min:s	Latitude (°)	Longitude (°)	Depth (km)	m_b ⊥	$\delta_{\perp}$ (km)	$\delta_{\parallel}$ (km)
1982/07/14	12:15:47.64	60.514	-153.670	157	5.0	1.4	2.1
1983/04/19	19:12:48.89	63.371	-149.957	112	5.1	0.0	3.8
1983/08/06	16:33:58.36	60.529	-153.129	137	5.4	1.8	-2.5
1983/09/26	23:52:52.91	57.445	-156.395	84	5.4	1.2	-0.6
1984/06/05	01:44:21.48	56.901	-157.262	94	5.3	0.4	-10.3
1987/07/25	01:11:48.86	60.155	-153.771	166	5.0	4.2	-6.1
1988/02/07	08:46:58.61	60.296	-152.972	137	5.6	-4.1	-24.1
1988/11/30	08:55:30.65	61.348	-152.270	143	5.5	-1.2	-26.1
1988/12/22	10:42:12.46	53.983	-166.244	76	5.4	2.9	9.3
1989/03/20	01:06:32.90	59.883	-153.692	127	5.1	-0.4	4.3
1990/05/09	02:38:57.4	57.502	-155.695	77	5.2	-9.7	0.7
1990/08/13	23:04:20.5	60.115	-152.006	88	5.3	NA	NA

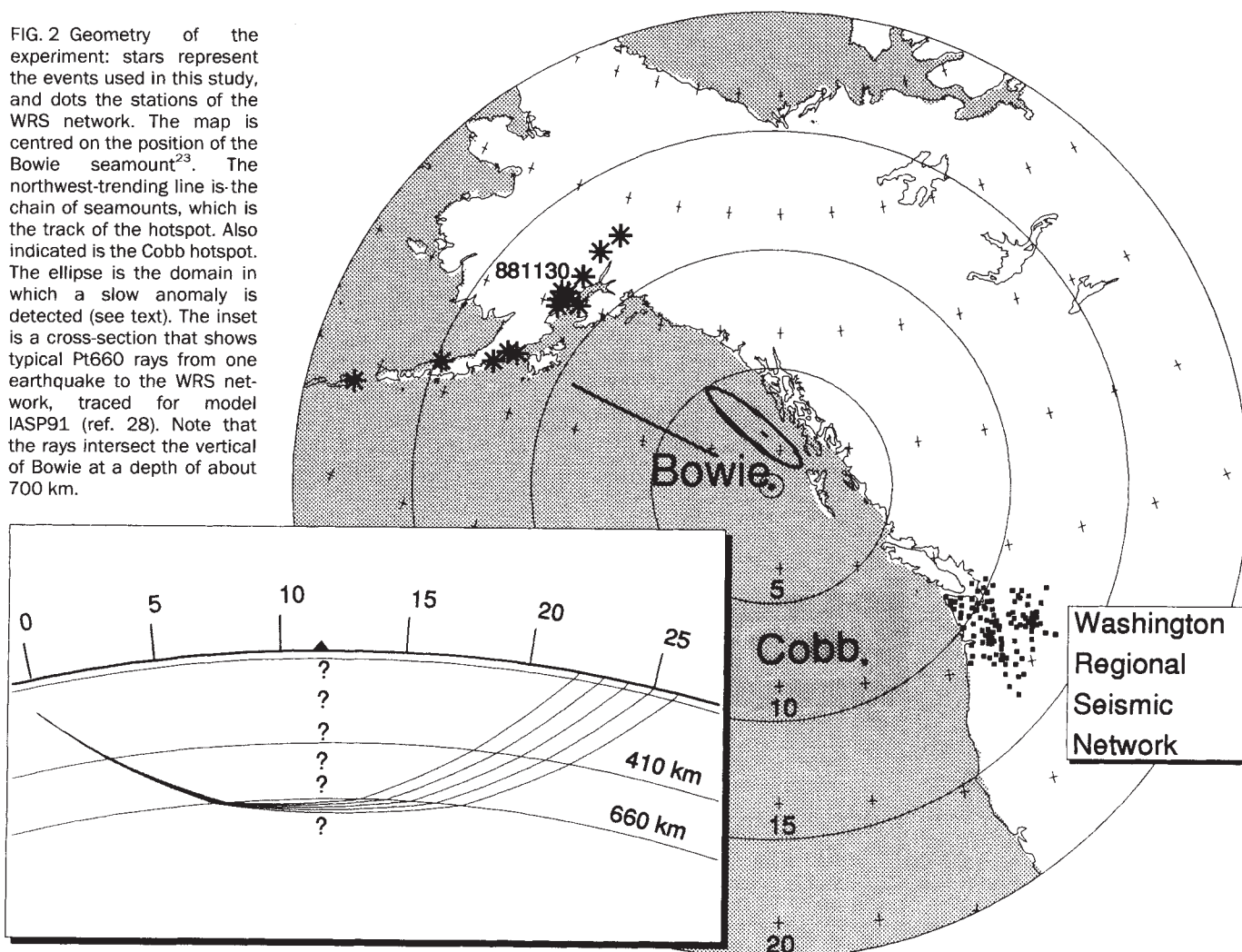
$\delta_{\perp}$ ($\delta_{\parallel}$) is the mislocation perpendicular (parallel) to the mean azimuth to WRSN, between the ISC hypocentre, and PDE USGS determination, which is the one listed here. The depth mislocation is included in the parallel mislocation. A systematic relocation of events, using ISC arrivals, the IASP91 model, and P, pP, pWP, PKPdf phases was recently done by E. R. Engdahl (personal communication). His results, available for four of our events, yield mislocations that are comparable with the ones reported here, except that the redetermined depths are closer to the PDE depths.

deviation less than 0.05 s. The stack (Fig. 3, top) reveals a simple waveform. At individual stations, the waveform often seems more complicated because in the distance range considered (21° to 26°), two waves arrive within a few seconds; the wave that has its bottoming point below the 660-km discontinuity (Pt660), and the wave that stays above it (Pn660). In the following, we keep only stations for which the Pt660 wave arrives first.

The second step is to calculate theoretical travel-times $t_{\text{theor}}^{e,s}$, using the radial model IASP91²⁸, and a residual time is formed: $t_{\text{res}}^{e,s} = t_x^{e,s} - t_{\text{theor}}^{e,s}$.

We then invert for a smooth single-valued function of residual travel-time against Δ_H . As much of the variation in travel-time residuals can be attributed to lateral heterogeneities in the crust and upper mantle beneath the stations, we perform a simultaneous inversion for the functional values and for station and

FIG. 2 Geometry of the experiment: stars represent the events used in this study, and dots the stations of the WRS network. The map is centred on the position of the Bowie seamount²³. The northwest-trending line is the chain of seamounts, which is the track of the hotspot. Also indicated is the Cobb hotspot. The ellipse is the domain in which a slow anomaly is detected (see text). The inset is a cross-section that shows typical Pt660 rays from one earthquake to the WRS network, traced for model IASP91 (ref. 28). Note that the rays intersect the vertical of Bowie at a depth of about 700 km.



event statics t_0^e and t_0^s (because the overall event origin times are also relatively unconstrained). We regularize the inversion by minimizing both the first and second differences of the function. Our inversion method is made robust by iteratively performing least-squares inversions, and down-weighting outliers (defined as data residuals greater than 1.5 standard deviation from the curve) at each iteration^{29,30}. The standard deviation is defined at each point on the curve by the residuals in a 1-degree interval assuming that the number of degrees of freedom is represented by the number of data points within each interval minus 1 (ref.

31). This is not strictly correct, as we have reduced the number of degrees of freedom through both the regularization of the problem and the inclusion of station and event statics. The end result of this process is to attribute as much data variance as possible to the static corrections while obtaining a curve with the least amount of structure necessary to explain the remaining travel-time residuals, as obtained from the expression $t_{\text{cor}}^{e,s} = t_{\text{res}}^{e,s} - t_0^e - t_0^s$.

Finally, corrected relative residuals are plotted as a function of Δ_H , the distance from the ray to the vertical of Bowie.

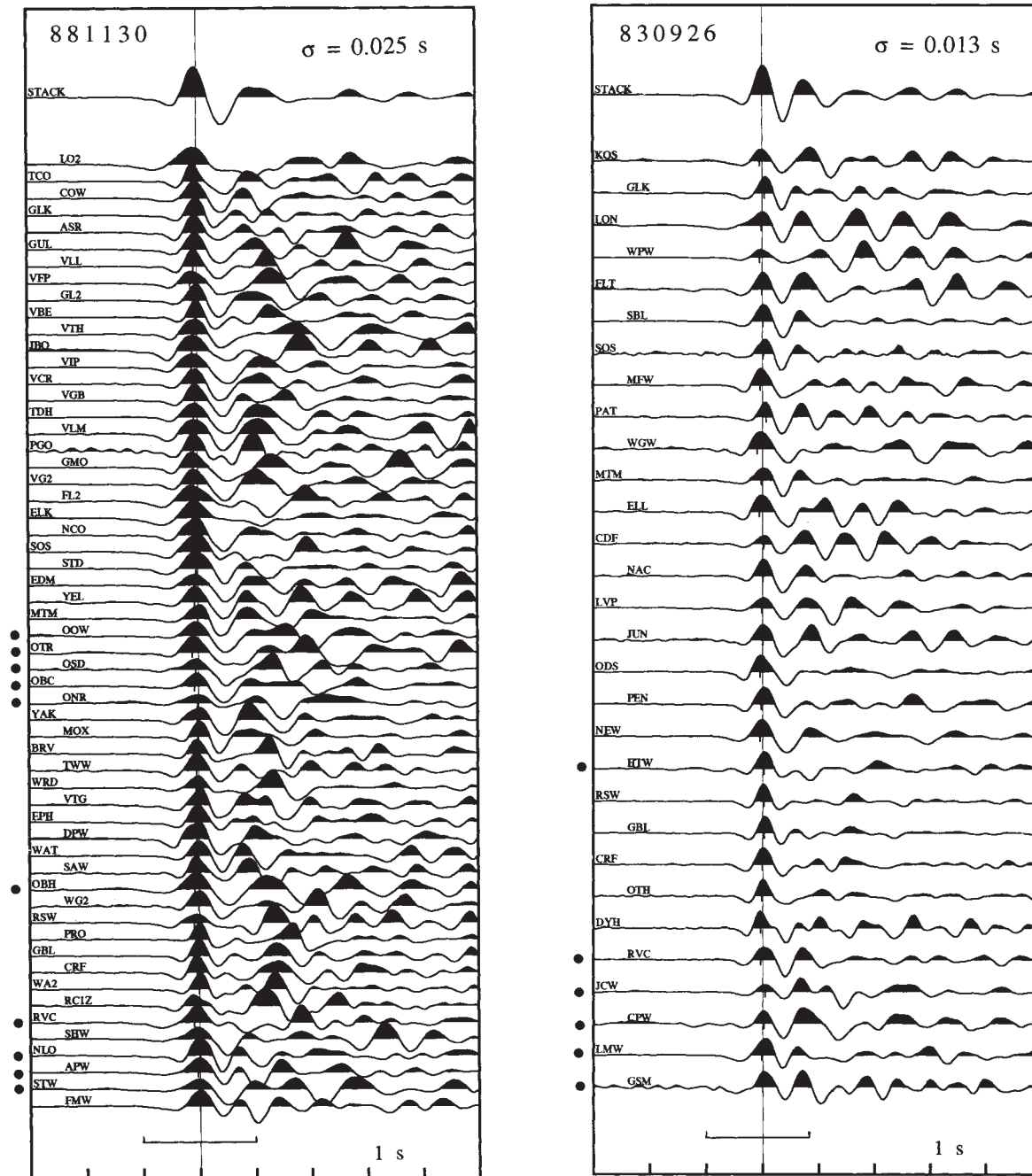


FIG. 3 Seismograms for events 881130 and 830926, as aligned through the multi-channel cross-correlation technique of VanDecar and Crosson²⁵. The traces have been low-pass filtered at 5 Hz. The top trace is the stack of the most similar waveforms, and is representative of the source wavelet. The bar at the bottom gives the time window used in

the cross-correlation. The vertical tic on each trace is the starting visual pick. The average timing standard deviation σ is indicated. For the stations marked with a dot, the first arrival is Pn660 instead of Pt660 (see text): these records have not been used in the rest of the analysis.

Detection of a mantle plume?

Figure 4a shows the plot of $t_{\text{cor}}^{\text{ss}}$ against Δ_H , for the 12 events. The scatter of the data is consistent with our estimated accuracy of about 0.1 s. Also drawn is the continuous curve of residuals against Δ_H as determined in the inversion process. The dashed lines give the standard deviation of the data around that curve. The bar at the bottom gives the size of the quarter-period Fresnel zone (defined as the region through which non-Fermat rays can pass and still arrive within a quarter-period from the Fermat ray) in the transverse direction to Δ_H . Although no clear pattern emerges around the expected plume position ($\Delta_H = 0^\circ$), a clear bell-shaped signal is visible around $\Delta_H = 1.5^\circ$. The delays reach a maximum of about 0.15 s, over a width of ~ 150 km. A smaller bump also appears around $\Delta_H = -1^\circ$, but this is produced by data from a single earthquake, and we do not think that it is really resolved.

The most straightforward, and exciting, interpretation of our results is that we have detected a plume beneath the Bowie hot-spot. In Fig. 4b, our smooth function, together with its 90% confidence limits, is superimposed on a plot of the delays predicted from the 'lower bound' and 'upper bound' models previously discussed. We see that the diameter of the anomaly we observe is close to the lower bound estimate, whereas the time-delay is about 1.5 times larger. As our inversion produces a 'minimal curve', the actual time-delay might be somewhat larger, as suggested by the individual trends of some events.

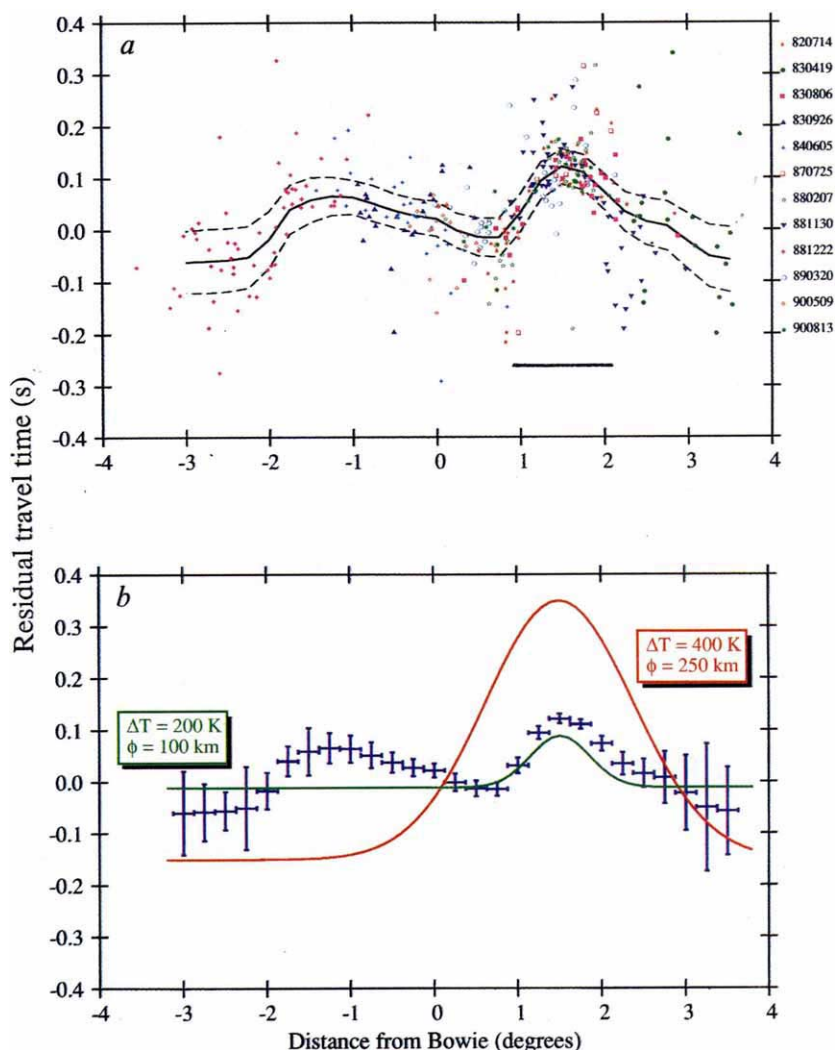
Before going further into the implications of this finding, we

should consider the likelihood of alternative explanations of our observations.

Near-source effects. For any given event, rays to WRSN leave the source within an azimuth window of 10° . If the bell-shaped anomaly is caused by a structure in the subducting plate, say 200 km thick, then the structure should be only 20 km wide. This is less than the distance between events 881130 and 880207, which do seem to show a coherent pattern. We could check, by removing one event at a time, that the position of the anomaly was not dependent on a single event. However, the amplitude of the anomaly is mostly constrained by the 881130 event, and to a lesser extent by the 880207 event. On the other hand, the small bump visible for Δ_H around -1° disappears entirely if the 881222 event is not included.

Near-receiver effects. A structure that could explain the bell-shaped delays would have to be present beneath a large part of the array. It should then also be seen for the other events, and in fact enter the 'station static correction'. The structure beneath the array being rather complex, some differential effect could remain, which could explain the observed pattern. To test this effect, we have done simple integrations and three-dimensional ray tracing in the tomographic model derived by VanDecar²⁶ for the region beneath the WRSN. We found that the computed delays were in good agreement with our 'statics', and that, for the considered azimuths, the differences between events were too small to explain our observations, although they certainly contribute to the scatter around our curve.

FIG. 4 a, Corrected relative residual travel-times as a function of Δ_H , the closest distance of the ray to the vertical of Bowie (counted positive to the northeast). Each plotted earthquake is given a symbol. Note the bell-shaped pattern of events 881130, 830806 and 880207. The solid curve is the best-fit function for residuals against Δ_H , and the dashed curves give the standard deviation of the data (see text). The horizontal bar at the bottom is the size of the quarter-period Fresnel zone. b, The best-fit curve shown in a (points with error bars) is compared with the expected delay curves for the two plume models discussed in the text (solid lines). The error bars are now the 90% confidence interval for deviations of the mean (see text). All curves have been given a zero mean over the range considered.



Anomalies elsewhere on the path. Our treatment assumes that the anomaly we are looking for is linked to the Bowie hotspot. This is very similar to the approach followed by Creager and Jordan³² for detecting slab penetration into the lower mantle. Obviously, our path coverage does not allow a full-size tomographic inversion to be done. However, our geometry does give some stereoscopic vision. We tried to evaluate where our 'image' was best in focus by running inversions for different trial positions of the Bowie plume along a line that could give an anomaly at $\Delta_H = 0^\circ$. We found the anomaly was best focused (maximum height ratio of scatter) in the elliptical domain shown in Fig. 2.

Inadequacy of the radial model. We found that model IASP91²⁸ could predict arrival times remarkably well. As we only deal with relative arrival times, it only matters that the slope and shape of the travel-time curve be accurate. This was apparently the case, as we did not find any correlation of residuals with epicentral distance for the Pt660 branch (most observations) but as the cross-over from Pn660 to Pt660 branch involves a strong change in slope, and is very sensitive to the exact model, distance and depth of the event, the residuals for the Pn660 branch had a scatter of up to 0.2 s, which correlated with epicentral distance, for a given event. All arrivals from the Pn660 branch were therefore excluded from the analysis. We also ran an inversion where Δ_H was replaced by the epicentral distance. We found no structure, down to ~ 0.05 s, except for the distances corresponding to event 881222. This event is the farthest one, and the relative station residuals might have to be slightly different for this distance.

Event mislocation. Two components should be distinguished: mislocation parallel to the average azimuth from the event to the network (together with depth mislocation), and mislocation perpendicular to the average azimuth. The two components play a very different role in our analysis.

If we were using absolute travel times, the 'parallel' mislocation $\delta_{\parallel}$ could be dangerous, as it creates a systematic pattern of delays as a function of azimuth, which could mimic our arcuate trend of residues versus Δ_H . But as we only deal with relative travel-times, the effect is in fact small. We have checked that parallel mislocations or depth mislocations of more than 50 km left the bell-shaped pattern of Fig. 4 almost unchanged. Such values largely exceed the plausible mislocations for that region (R. D. van der Hilst, personal communication, see also Table 1).

The effect of the 'perpendicular' mislocation $\delta_{\perp}$ is that the wavefronts arrive with an angle different from the expected one,

thereby producing a linear trend in the plot of Fig. 4, for a given event. The effect could be pronounced, as a perpendicular mislocation of 20 km produces a linear trend of 0.2 s across the array. By considering rays that depart almost perpendicular to the strike of the Alaskan subduction zone, we reduce this effect. It is well known that the component of mislocation parallel to the strike of the slab is much less than the component perpendicular to the slab. Perpendicular mislocations should not exceed a few kilometres.

Inversion artefact. The structure in Fig. 4 could result from trying to fit a smooth curve to randomly scattered points. We checked that this was not the case by running an inversion in which all Δ_H had been randomly permuted, while events and stations were kept unchanged. No structure was visible in the curve obtained, down to a level of about 0.05 s.

Plume characteristics

At this stage, the detection of the plume cannot be considered definitive. However, we believe that our analysis is the best attempt so far, and is the best that can be done with current data and techniques. The most plausible explanation of the bell-shaped signature in Fig. 4 does seem to be that there is a mantle plume (or diapir) beneath Bowie. If true, this observation has several implications. The diameter of the plume is about 150 km. Its core is at least 1.5% slower than the surrounding mantle. This translates into a temperature contrast of at least 300 K, unless velocity is reduced by additional effects (partial melting or anisotropy). As the rays that we analyse intersect the Bowie plume below the 660 km discontinuity, we are led to conclude that the plume originates below that discontinuity, in the lower mantle. Finally, at ~ 700 km depth, the centre of the plume is 150 km northeast of the position of the Bowie seamount. Age data suggest that the Bowie hotspot is at present ~ 100 km southeast of the seamount²⁴. Our data indicate that it could be even farther away from the Bowie seamount, or else that the plume is not vertical.

Our results could be tested and extended in a number of ways. The numerous seismic stations on the western margin of North America should make it possible to retrieve longer profiles, and investigate how the anomaly evolves with depth. A detailed analysis of amplitudes and waveforms would put bounds on the internal structure of the plume. Our synthetic seismograms suggest that the anomaly to be expected for the Hawaiian plume should remain detectable as far away as North America. $\square$

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1. Morgan, W. J. *Nature* **230**, 42–43 (1971).
2. Wilson, J. T. *Can. J. Phys.* **41**, 863–870 (1963).
3. McKenzie, D. P., Roberts, J. M. & Weiss, N. O. *J. Fluid Mech.* **62**, 465–538 (1974).
4. Anderson, D. L. *Theory of the Earth* (Blackwell, Boston, 1989).
5. Schilling, J. G. *Nature* **352**, 397–403 (1991).
6. Watson, S. & McKenzie, D. P. *J. Petrol.* **32**, 501–537 (1991).
7. Loper, D. E. & Stacey, F. D. *Phys. Earth planet. int.* **33**, 304–317 (1983).
8. Iyer, H. M. et al. *Geol. Soc. Am. Bull.* **92**, 792–798 (1981).
9. Hadley, D. M., Stewart, G. J. & Ebel, J. E. *Science* **193**, 1237–1329 (1976).
10. Zhang, Y.-S. & Tanimoto, T. *Nature* **355**, 45–49 (1992).
11. Olson, P. & Singer, H. J. *Fluid Mech.* **158**, 511–531 (1985).
12. Kanasewich, E. R., Ellis, R. M., Chapman, C. H. & Gutowski, P. R. *Nature* **239**, 99 (1972).
13. Wright, C. J. *Geophys. Res.* **80**, 1915–1919 (1975).
14. Hirahara, K. *J. Phys. Earth* **25**, 393–417 (1977).
15. Humphreys, E., Clayton, R. W. & Hager, B. H. *Geophys. Res. Lett.* **11**, 625–627 (1984).
16. Spakman, W. *Geophys. J. int.* **107**, 309–332 (1991).
17. Davies, G. F. *J. geophys. Res.* **93**, 10467–10480 (1988).
18. Sleep, N. H. *J. geophys. Res.* **95**, 6715–6736 (1990).
19. Kumazawa, M. & Anderson, O. L. *J. geophys. Res.* **74**, 5961–5972 (1969).
20. Peltier, W. R. & Solheim, L. P. *Geophys. Res. Lett.* **19**, 321–324 (1992).

21. Wielandt, E. in *Seismic Tomography* (ed. Nolet, G.) 85–98, (Reidel Dordrecht, 1987).
22. Bostock, M. G., VanDecar, J. C. & Snieder, R. *Bull. seism. Soc. Am.* **83**, 756–779 (1993).
23. Morgan, W. J. *Am. Assoc. Petrol. Geol. Bull.* **56**, 203–213 (1972).
24. Turner, D. L., Jarrard, R. D. & Forbes, R. B. *J. geophys. Res.* **85**, 6547–6556 (1980).
25. VanDecar, J. C. & Crosson, R. S. *Bull. seism. Soc. Am.* **80**, 150–169 (1990).
26. VanDecar, J. C. thesis, Univ. of Washington (1991).
27. Duncan, R. A. & Clague, D. A. in *The Ocean Basins and Margins*, Vol. 7A, *The Pacific Ocean* (eds Naim, A. E. M., Stehli, F. G. & Uyeda, S.) 89–121 (Plenum, New York, 1985).
28. Kennett, B. & Engdahl, E. R. *Geophys. J. int.* **105**, 429–465 (1991).
29. Egbert, G. D. & Booker, J. R. *Geophys. J. R. astr. Soc.* **87**, 173–194 (1986).
30. Huber, P. J. *Robust Statistics* (Wiley, New York, 1981).
31. Snedecor, G. W. & Cochran, W. G. *Statistical Methods* (Iowa State Univ. Press, Ames, 1967).
32. Creager, K. C. & Jordan, T. H. *J. geophys. Res.* **89**, 3031–3049 (1984).

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Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast

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A novel genetic selection in yeast has been used to isolate a complementary DNA for the transcriptional activator, Olf-1, which binds to the regulatory sequences of several olfactory-specific genes. The Olf-1 protein, expressed exclusively in the olfactory receptor neurons and their precursors, contains a new helix-loop-helix motif and functions as an apparent homodimer. Olf-1 may be the first member of a family of related proteins that may direct cellular differentiation in a variety of neuronal tissues.

THE mammalian olfactory system has the remarkable ability to detect odorants with high sensitivity and specificity. The initial events in the olfactory signal transduction pathway occur in the specialized cilia of the sensory neurons¹. Unlike other neurons, these olfactory sensory cells are continually replaced throughout adult life². This continued neurogenesis makes the olfactory system an excellent model for the genetic and developmental processes that accompany neuronal differentiation.

Cells within the olfactory epithelium follow a highly ordered developmental programme resulting in the high-level expression of gene products essential for odorant signal transduction. Basal cells, which lie near the basal lamina of this pseudostratified epithelium, give rise to progenitors of the mature sensory neurons as well as non-neuronal support cells. The neuronal precursor cells follow a developmental programme that directs their differentiation into mature olfactory neurons. During this process each neuron extends a dendritic process to the surface of the epithelium and a single axon to the olfactory bulb where the mature neuron synapses with second order neurons. At about this time, the mature neuron expresses several olfactory-specific genes, some of which appear to mediate the odorant signal transduction cascade. Evidence supporting the involvement of a G-protein-coupled receptor pathway in odorant signal transduction has come from the isolation of olfactory-specific components that correspond to each step in the pathway. These include $G_{\alpha_{olf}}$ (ref. 3), type III adenylyl cyclase⁴ and a cyclic-nucleotide-activated ion channel, OcNC (refs 5,6). Additional olfactory neuron-specific genes have also been identified whose functions remain unknown⁷.

The establishment of the mature olfactory neuronal phenotype probably results from the coordinated expression of olfactory-specific genes. Experiments in other systems have suggested that tissue-specific transcription factors are responsible for turning on tissue-specific genes through interactions with specific DNA sequences. The myogenic helix-loop-helix family of transcription factors trigger the conversion of several cell lines into myoblasts through sequence-specific interactions with muscle-specific genes⁸⁻¹⁰. To understand the regulation olfactory neuronal-specific gene expression, we have investigated the structure of six olfactory-specific genes (M.M.W. and R.R.R., manuscript in preparation). Our studies indicate that each of these genes contains at least one binding site for the DNA-binding protein, Olf-1. The binding of an olfactory-specific factor, Olf-1 was first described in the OMP gene³⁸. The Olf-1 activity is detected in nuclear extracts from nasal epithelium and is absent from nuclear extracts of a variety of other tissues. The transcriptional activator Olf-1, may be the

critical protein factor involved in activating the expression of the signal transduction components and other genes of the olfactory system.

We have identified a cDNA clone encoding the Olf-1 binding activity using a genetic selection in yeast. The deduced amino-acid sequence of Olf-1 is predicted to contain two helical repeats with similarity to the helix-loop-helix class of transcription factors, and may represent the first identified member of a novel subclass containing this protein motif. The Olf-1 protein is restricted to the mature olfactory neurons and their progenitors in olfactory epithelium. Olf-1 is the first transcriptional activator identified likely to direct the coordinate expression of cell-specific genes in a neuronal system.

Cloning of a specific DNA-binding protein

We have characterized the promoter regions for three genes (G_{olf} , type III adenylyl cyclase and the olfactory cyclic nucleotide gated channel) involved in the olfactory second messenger cascade and three additional genes (OMP⁷, 50.06, and 50.11) specifically expressed by olfactory neurons (M.M.W. and R.R.R., manuscript in preparation). A *cis*-acting regulatory sequence present in each of these genes was identified and is described in detail elsewhere. Briefly, an activity, Olf-1, detectable only in olfactory nuclear extracts was found to bind to a site 150 nucleotides upstream of the OcNC transcriptional start site. Using this site in gel mobility assays, sites were identified in each of the other olfactory-specific genes. DNA binding sites (U sites) for other factors present in many tissues exist in some of these promoter regions. The sequence of each of the binding sites as well as their position and orientation in the olfactory neuron-specific genes is shown in Fig. 1.

The low abundance of sensory neurons in olfactory tissue preparations suggested that purification of the Olf-1 binding activity by biochemical methods would prove difficult. Although several DNA-binding proteins have been cloned by screening recombinant expression libraries with radiolabelled concatemeric binding sites^{11,12}, attempts to screen olfactory cDNA expression libraries with the OcNC Olf-1 binding sites were unsuccessful. We developed a novel cloning strategy using a genetic selection of olfactory cDNAs encoding proteins capable of activating a reporter construct in yeast (Fig. 2). Conceptually, we wished to identify plasmids in a cDNA library that could direct the expression of a protein that binds the Olf-1 site. The olfactory cDNA library was expressed in yeast as a translational fusion with a GAL4 transactivating domain. The reporter construct consisted of three copies of the OcNC Olf-1 binding site adjacent to a low activity promoter directing *HIS3*

gene expression. The presence of this reporter is insufficient to rescue the *his3*⁻ phenotype of the parent yeast strain. Yeast that harbours a GAL4-olfactory fusion protein capable of binding the Olf-1 sites present in the reporter will activate transcription and increase levels of *HIS3*. The use of the selectable marker *HIS3* enabled direct selection of candidate clones. Others have used yeast expression systems to identify subunits involved in

protein-protein interactions¹³. The system to identify specific DNA-protein interactions described here is fundamentally similar to the two-hybrid system¹⁴ and is conceptually complementary to previously described binding-site selection schemes in yeast¹⁵.

An olfactory cDNA library of 3.6 million clones was constructed in the yeast expression vector and transformed into

FIG. 1 The genomic structure, location of Olf-1 sites within olfactory specific genes, and sequences of Olf-1 sites. The olfactory cyclic nucleotide channel (OcNC), the olfactory neuron-specific G protein (*G_{olf}*) and type III adenylyl cyclase (AC-III) are components of the odorant detection pathway whereas olfactory marker protein (OMP) and 50.06 are of unknown function. In each map, the exons of the gene are represented by black bars; the start site for transcription is indicated by an arrow and Olf-1 (striped boxes) and U sites (unpublished data) (bound by a distinct, broadly expressed DNA-binding site, shaded boxes) are indicated. The top strand sequences of the double-stranded oligonucleotides corresponding to Olf-1 sites in the indicated genes are listed with the Olf-1 site in bold. Mutant oligonucleotides used in DNA binding studies are shown at the bottom with the single base changes underlined.

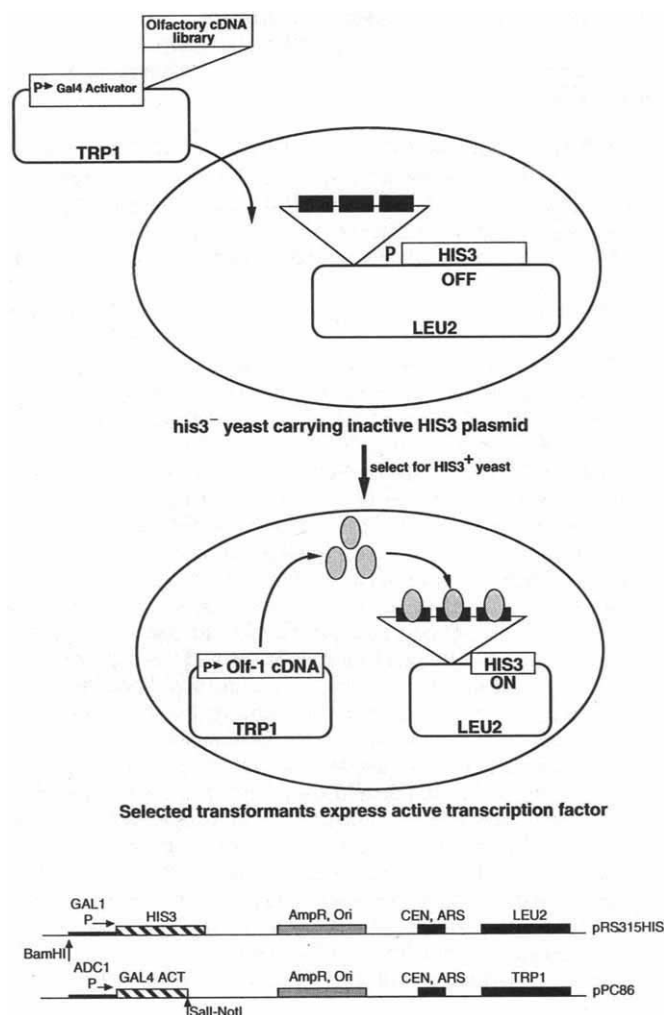
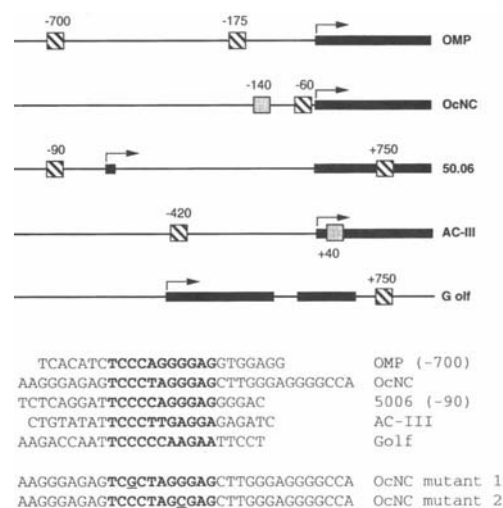


FIG. 2 Scheme and vectors used for cloning of the Olf-1 DNA-binding protein by selection in yeast. The yeast strain yWAM2 was transformed with the *HIS3*-containing plasmid p59.7 to generate the illustrated recipient strain for the expression library. The plasmid in this strain was maintained extrachromosomally by selection for LEU2. This yeast strain was then transformed with an olfactory cDNA library and plated directly on media lacking histidine. Histidine prototrophs that grew in the absence of tryptophan and leucine presumably arose by transactivation mediated by an olfactory cDNA and were further analysed. Bottom, A more detailed schematic of the reporter vector and expression vector used with the unique restriction sites used for insertion of Olf-1 sites and olfactory cDNA, respectively. METHODS. The yeast strain yWAM2 (*MATα Δgal4 Δgal80 URA3::GAL1-lacZ lys2801amber his3-Δ200 trp1-Δ63 leu2ade2-101ochreCYH2*; provided by P. Hieter and W. Michaud) was derived from PCY2 (ref. 27). The yeast reporter plasmids were constructed by insertion of annealed complementary oligonucleotides containing Olf-1 sites into the *Bam*HI site of the vector pRS315HIS. The plasmid pRS315HIS was generated by inserting a *Bam*HI-SalI fragment containing the GAL1 minimal promoter adjacent to *HIS3* from plasmid HR307a¹⁵ into pRS315²⁸. The p59.7 reporter construct used in the selection for Olf-1 binding activity contained three copies of the OcNC Olf-1 binding site. The sites were concatamerized by ligating annealed oligonucleotides to yield *Bam*HI- and *Bgl*III-compatible ends. Yeast transformed with p59.7 were maintained by growth in supplemented synthetic dextrose media (SD) without leucine. Olfactory epithelium cDNA generated from 3-week-old Sprague-Dawley rats was directionally cloned into the *Sal*I-NotI sites of the TRP-1 marked yeast expression vector pPC86²⁷ using the Superscript cDNA synthesis system (BRL). The ligation products were electroporated into *E. coli* strain DH10B, and the resultant transformants (3.6×10^6 with an average insert size of 1.5 kb) plated onto LB/carbenicillin plates at a density of 1×10^5 colonies per plate. The plasmid library DNA was purified over Qiagen columns after scraping the cells from the plate, and used for transformation into yeast by the PEG/lithium acetate method²⁹. All yeast transformations were done with an efficiency of $>10^4 \mu\text{g}^{-1}$ vector. Transformations with the olfactory cDNA library were plated directly on SD media without histidine (but including leucine and tryptophan) and incubated for 3 days at 30 °C. Transformation efficiency was determined by plating an aliquot of the transformation mixture onto SD media minus leucine and tryptophan.

a *his3⁻* yeast strain carrying the *HIS3* reporter plasmid. Two million transformants were plated on media lacking histidine and 30 *HIS⁺* colonies were isolated. One colony grew on media lacking tryptophan, indicating that it contained the *TRP1*-marked *GAL4*-olfactory cDNA expression plasmid. This plasmid (Y11) was recovered by transformation into *Escherichia coli*. Y11 is also able to activate *HIS3* transcription in yeast containing a similar reporter plasmid with two copies of an Olf-1 site from the 50.06 gene (Fig. 1). In contrast, the Y11 plasmid fails to rescue the *HIS⁺* phenotype of cells containing the parental *HIS3* vector. These results are consistent with a specific interaction between a protein encoded by Y11 and the Olf-1 sites that leads to transcriptional activation of *HIS3*.

Y11 encodes an unusual helix-loop-helix protein

Nucleotide sequence analysis of the Y11 insert revealed an open reading frame of 570 amino acids which initiated from the first methionine in good context for translation initiation¹⁶ (Fig. 3). An additional ATG codon in poor context for translation initiation precedes the predicted start codon. Analysis of genomic DNA and RACE-PCR products (data not shown) demonstrates that the presumed initiation methionine is preceded in the full-length mRNA by an in-frame stop codon. As expected, the Y11 cDNA insert was in frame with the *GAL4* transactivator domain (*GAL4TA*), enabling Y11 to direct expression of a fusion protein between the *GAL4TA* and the novel 570-amino-acid polypeptide.

The carboxy-terminal region (residues 441–570) of the Y11 protein is extraordinarily rich in serine (23%) and proline (12%) and may serve as a transactivation domain¹⁷. In addition, residues 228–232 (Arg-Arg-Ala-Arg-Arg) fit the consensus for a nuclear localization signal. Secondary structure analysis of the Y11-encoded protein predicted that amino acids 354–391 form an α -helix followed by a turn or loop and a second α -helical region. These helices, nearly identical at the amino-acid level, shared modest similarity to the second helix of the helix-loop-helix (HLH) family of transcription factors^{18,19}. Specifically, this region of Y11 was identical at several hydrophobic residues that have been conserved among the HLH transcription factors and are believed to participate in inter- and intramolecular coiled-coil interactions²⁰. The Y11 protein displayed no homology to the first helix of the HLH family. Though Y11 protein contains several basic residues upstream of the helices, it lacks highly conserved basic residues immediately upstream of the HLH region present in members of the HLH family which activate transcription^{21,22}. These observations suggest that Y11 represents the first member of a novel class of proteins containing a repeated helix-loop-helix (rHLH) motif. In light of the properties of the Y11 cDNA, we conclude that Y11 is likely to encode the Olf-1 binding activity. The data presented below strengthens this conclusion.

Previous biochemical characterization of the Olf-1 binding activity suggested that the protein was confined to the olfactory epithelium. To establish that Y11 encoded the expected olfactory-specific binding activity, the level of Y11 messenger RNA expression in six tissues was examined by reverse transcriptase polymerase chain reaction (RT-PCR). A PCR product of the expected size was detected exclusively in template derived from olfactory epithelium (Fig. 4a). This expression pattern paralleled that determined for Olf-1 binding³⁸.

The DNA binding properties of the Y11-encoded protein were assessed by electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides containing Olf-1 sites from the five olfactory-specific genes were specifically shifted by protein translated in a reticulocyte lysate (Fig. 4b). In contrast, two oligonucleotides containing single base changes in the OcNC site, known to eliminate the ability of native Olf-1 protein to bind DNA, failed to produce the shifted product. Polyclonal

antisera raised to amino acids 360–570 of Y11 specifically recognized the protein-DNA complex present in nuclear extracts from olfactory epithelium. When the Y11 antiserum was mixed with preformed Olf-1-DNA complexes, a super-shifted band of slower mobility representing a ternary complex of Olf-1 protein/labelled DNA probe/antibody was observed (Fig. 4c). The preimmune serum did not affect the mobility of the Olf-1 complex. Moreover, the Y11 antisera did not supershift an unrelated DNA-protein complex (Fig. 4c).

The consensus Olf-1 binding site (TCCYRRGGAG) is nearly symmetric implying that Olf-1 may bind to its site as a dimer. Furthermore, the similarity of the rHLH region of Olf-1 to the basic HLH (bHLH) domains, shown to mediate dimerization in the MyoD family, suggested that Olf-1 participates in stable protein-protein interactions. C-terminal truncated forms of Olf-1 that include the rHLH region retained their ability to bind DNA but resulted in a faster mobility complex than was observed with the full-length protein. The cotranslation of mRNA derived from truncated and full-length Olf-1 cDNA in reticulocyte lysates led to the formation of a

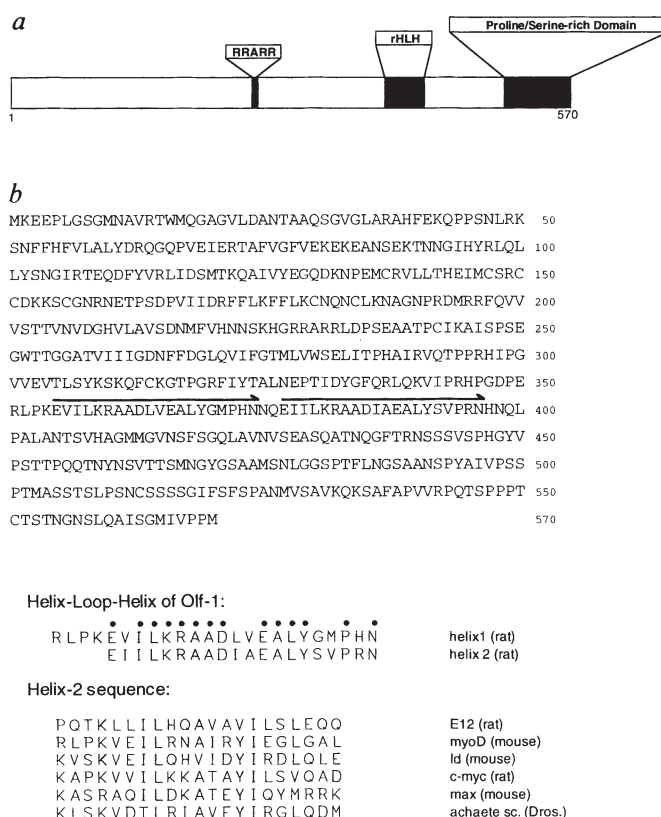


FIG. 3 Sequence and domain structure of Y11 and homology to the helix-loop-helix class of transcription factors. Schematic diagram (a) and deduced amino-acid sequence (b) of Y11 are shown on top. The locations of the putative nuclear localization signal (RRARR), repeat helix-loop-helix (rHLH) and the serine/proline-rich activation domain are indicated. The sequence of the repeated helix-loop-helix motif predicted by both the Chou-Fasman and Robson-Garnier secondary structure algorithms^{30,31} is denoted by arrows. Comparison of the rHLH sequence of Y11 to the sequences of the second helix from members of the HLH family is displayed^{18,19}. Residues that are identical in both helices of the predicted rHLH domain are indicated with dots and shaded when a single amino acid appears in at least 5 of the 8 sequences.

METHODS. Total DNA was prepared from yeast Y11 and used to transform *E. coli*. The rescued plasmid Y11 was sequenced on both strands using a combination of exonuclease III deletions and synthetic primers with Sequenase (USB).

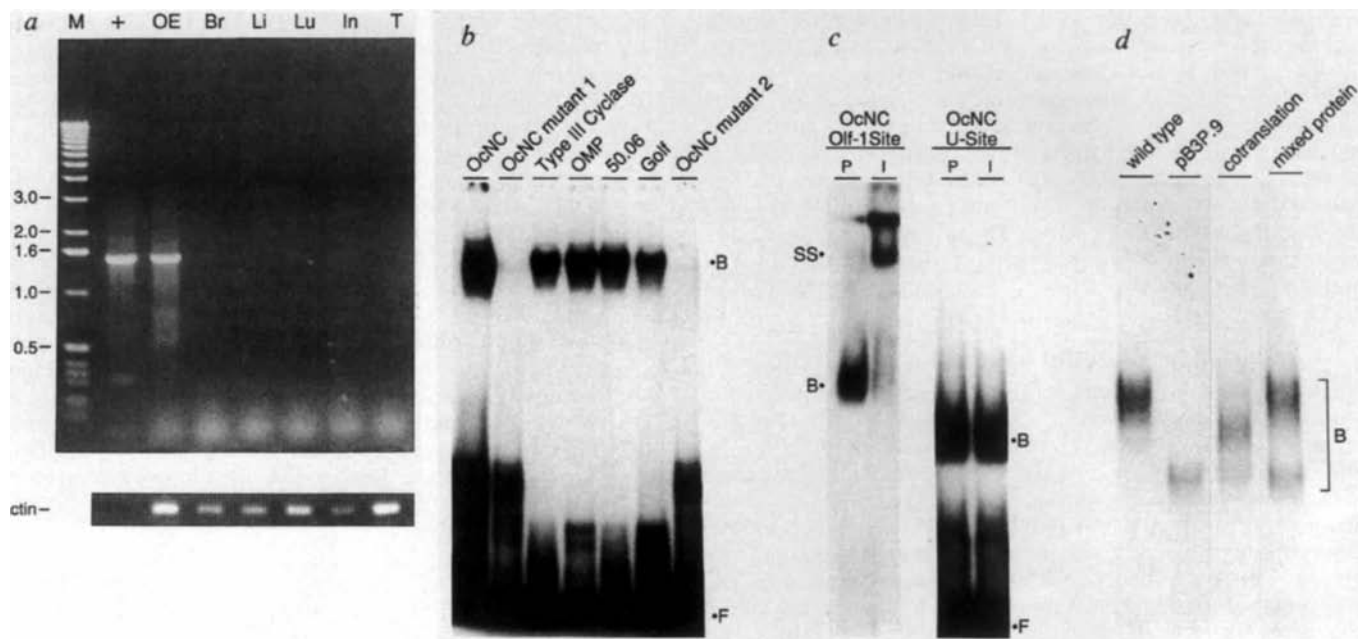
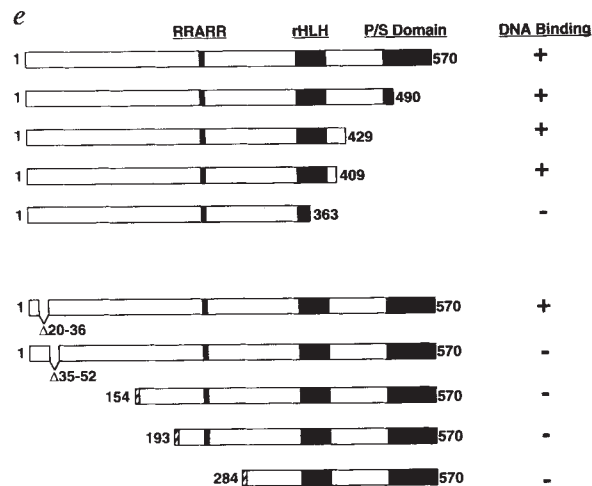


FIG. 4a, Expression levels of the Y11 mRNA in various tissues. Y11-specific oligonucleotides were used for RT-PCR on first strand cDNA and the PCR products analysed by gel electrophoresis as previously described³². M, molecular mass markers; +, 1 ng purified Y11 plasmid as a positive control; OE, olfactory epithelium; Br, brain; Li, liver; Lu, lung; In, intestine; T, testis. b, Gel shifts using *in vitro* expressed protein programmed from Y11. Protein produced by *in vitro* transcription and translation of the Y11 cDNA was incubated with radiolabelled probes encoding Olf-1 sites and analysed by EMSA. The sequence of each of the oligonucleotides sites is shown in Fig. 1. c, Antibodies to the Y11-encoded protein supershifts the Olf-1–DNA complex. Binding reactions were done by combining olfactory nuclear extracts with ³²P-labelled Olf-1 site from OcNC or a sequence (Fig. 1) present in the OcNC promoter which binds to a broadly expressed nuclear factor (U box - 5' GATCCTGTCTGGAAGTCTGCCAGCATTTAA) (M. M. W. and R. R. R., manuscript in preparation). Pre-immune serum (P) or polyclonal antiserum JH865 (I) was added to each reaction and the mixture analysed by EMSA. Free Olf-1 oligonucleotide probe was run off the gel. d, Olf-1 binds to DNA as a multimer. The full-length Olf-1 protein (wild type), truncated Olf-1 (pB3P-9) and cotranslated or post-translationally mixed full-length and truncated Olf-1 were incubated with labelled OcNC Olf-1 oligonucleotide and used for EMSA. The bracket indicates the position of bound complexes in each of the lanes e, Deletional analysis of the Olf-1 protein. Olf-1 protein was generated by *in vitro* transcription/translation of appropriate templates resulting in products whose primary structure are illustrated. The ability of truncated and deleted proteins to bind radiolabelled Olf-1 sites was assessed in gel mobility shift assays. The positions of the N- and C-terminal truncations and internal deletion endpoints are shown. N-terminal deletions were fused to a synthetic adaptor, depicted by the hatched box, to provide an initiator methionine.

METHODS. RT-PCR was done on total RNA from the indicated tissues using primers designed to amplify Y11 message. The oligonucleotides MW125 (5' CCACAGTCAACGTGGAT amino acids 203–208) and MW122 (5' AGTCTTATTAGAGTGGC 3' untranslated region) were used in PCR reactions with 5 μM primer and 1 ng oligo-dT-primed cDNA template per 20 μl reaction. Reaction conditions were 94 °C for 45 s, 56 °C for 1 min, and 72 °C for 2 min for 35 cycles. The insert of Y11 was subcloned into Bluescript KS⁺ to create the plasmid pB3P, and protein for gel shifts were prepared by transcribing sense RNA from linearized pB3P and translating 1 μg of the RNA in 25 μl of rabbit reticulocyte lysate as described by the manufacturer (Promega). A 2 μl aliquot of the translation mix was added to a 20 μl binding reaction (10 mM HEPES (pH 7.9 at 4 °C), 10 mM MgCl₂, 10% glycerol, 50 mM KCl, 0.5 mM DTT, 50 μg ml⁻¹ dIdC, 50 μg ml⁻¹ salmon sperm DNA, and 50 pg of ³²P-labelled oligonucleotide probe (2,000 Ci mmol⁻¹). After a 10 min incubation on ice, the mixture was electrophoresed on a 6% polyacrylamide gel (59:1 acrylamide:bisacrylamide) in 0.25 × TBE (1 × TBE is 90 mM TrisBorate pH 8.3, 2 mM EDTA) at 4 °C. The gel was dried and exposed to film for 6 h. Protein from olfactory nuclear extracts³³ were added to 20-μl binding reactions to a final protein concentration of 0.1 mg ml⁻¹ and incubated for 10 min on ice. Serum (1 μl) was added to the mixture and the reaction continued for 10 min on ice. Immune serum was obtained from rabbits injected with GST-Y11 fusion protein prepared from *E. coli* BL21 cells carrying a *Bgl*II–*Hin*III fragment of B3P, corresponding to amino acids 363 to 570, ligated into pGEX-1³⁴. Protein was eluted from glutathione-agarose beads by incubation in 50 mM Tris–HCl pH 8.0, 5 mM reduced glutathione³⁵. Full-



length Olf-1 was produced in reticulocyte lysate as described above. Truncated Olf-1 protein was generated by translating RNA from a T3 RNA polymerase transcription from linearized plasmid pB3P.9 which encodes amino acids 1–430 of Y11. pB3P.9 was derived by subcloning into Bluescript the *Sall*–*Sac*II fragment of an exonuclease III-generated 3' deletion of Y11. DNA templates for generating C-terminal truncations were prepared by linearizing pB3P with a restriction enzyme which cleaved within the coding region of the Olf-1 protein or by cloning partial exonuclease III deletions of the 3' end of the cDNA. Plasmids used to synthesize N-terminal-deleted proteins were derived from partial clones isolated from an olfactory cDNA library. The *Eco*RI inserts of these clones were ligated to a *Sall*–*Eco*RI adaptor encoding an in-frame initiator methionine followed by nucleotides encoding the sequence VPPKKRKRKVL (hatched leader sequence), digested at a unique *Hind*III site in the 3' untranslated region, and the *Sall*–*Hind*III fragment subcloned into a plasmid capable of directing *in vitro* transcription. DNA that coded for the internal deletion of residues 20 to 36 was created by digestion of pB3P with *Nar*I and *Sma*I, blunting the ends with the large fragment of DNA polymerase I, and recircularizing with DNA ligase. This procedure resulted in the substitution of a glycine at the deletion junction. An internal deletion of residues 35 to 52 was generated by partial exonuclease III digestion of *Sma*I-cleaved pB3P. All deletion junctions were sequenced. The yield and size of the protein products generated from these constructs were verified by SDS–PAGE analysis of ³⁵S-labelled translation products.

protein–DNA complex of intermediate mobility by EMSA (Fig. 4d). The intermediate position of this complex is consistent with the conclusion that Olf-1 binds DNA as a homooligomer. When the two proteins were mixed after translation, the novel complex was not produced.

The divergence of the Olf-1 helix–loop–helix region from the consensus bHLH and the absence of a conserved basic region in Olf-1 implied that Olf-1 interacted with DNA in a fundamentally different manner. Deletional analysis of the Olf-1 protein provided evidence that the rHLH region was necessary but not sufficient for DNA binding (Fig. 4e). Specifically, sequence contained within an 18 amino-acid deletion, situated more than 350 amino acids away from the rHLH, were necessary for binding DNA. This is in contrast to myoD, where a 68-amino-acid region containing the bHLH region of the protein is sufficient for high-affinity DNA binding and transcriptional activation^{10,23}. Given the modular nature of DNA-binding proteins, we hypothesize that the rHLH region of Olf-1 might

mediate oligomerization, whereas the N-terminal domain might be responsible for sequence-specific contacts with DNA.

Olf-1 localized to nuclei of olfactory neurons

To determine which cells in rat olfactory epithelium express the Olf-1 protein, we used immunohistochemistry with the Olf-1 antisera. Immunoreactive material was detected in the nuclei of mature olfactory neurons as well as in the region of the olfactory epithelium closer to the basal lamina which contains a high fraction of immature neuronal precursors^{2,24,25} (Fig. 5). In contrast, the nuclei of sustentacular cells and the most basal cell population did not stain with the Olf-1 antibody. This was seen most clearly when the nuclei were revealed with the DNA-binding dye, DAPI. The presence of Olf-1 immunoreactivity in the mature olfactory neurons was consistent with the high level of expression of putative target genes in these cells. The possibility that Olf-1 immunoreactivity extends to the neuronal precursors suggests that Olf-1 may play an early role in directing

FIG. 5 Pattern of Olf-1 immunoreactivity in adult olfactory epithelium. Sections were incubated with the Olf-1-specific antiserum (top) or preimmune serum (bottom) and visualized with the Vectastain ABC Elite kit. SUS, Sustentacular cell nuclei; ORN, olfactory receptor neurons; BASAL, basal cells. To reveal the location of all the cell nuclei in the epithelium, sections were stained with the fluorescent DNA-binding dye DAPI (1 $\mu\text{g ml}^{-1}$) before mounting. Note that the diaminobenzidine immunoreaction product quenches the DAPI fluorescence and serves to identify the immunoreactive cell nuclei under bright field (left) and ultraviolet illumination (right).

METHODS. Adult male SD rats were anaesthetized and perfused. Paraformaldehyde-fixed tissue from olfactory areas were embedded in paraffin and sectioned at 8 μm . Immunohistochemistry was as described with the ABC kit (Vector Labs) except that sections were blocked with 10% goat serum. The immune serum JH865 was preabsorbed with an excess of GST-bound glutathione beads.

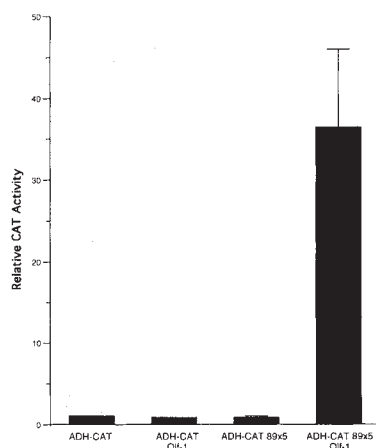
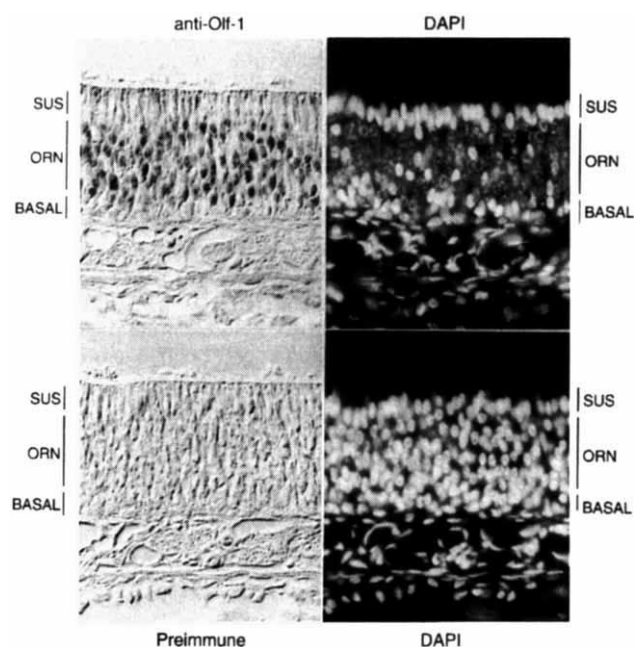


FIG. 6 Functional expression of Olf-1 in HEK 293 cells. Cells were cotransfected with a basal promoter CAT reporter construct (ADH-CAT) or a CAT reporter downstream of five copies of the distal 50.06 Olf-1 site (ADH-CAT89X5) in the presence or absence of a plasmid expressing Olf-1. CAT activity, normalized for protein concentration, was determined from four independent transfection experiments.

METHODS. Cotransfections were essentially as described³⁶. Each transfection included 1 μg CAT vector, 5 μg expression vector, 5 μg salmon sperm DNA and, when specified, 50 ng Olf-1 expression plasmid per 6-cm dish. The reporter construct pADHCAT was constructed by ligating into Bluescript the *Hind*III fragment of pD-33CAT³⁷, containing the minimal promoter of the *Drosophila* alcohol dehydrogenase gene fused to CAT. Concatemerized oligonucleotides (Fig. 1; 5006-710) were cloned into the *Bam*HI site adjacent to the ADH sequences of pADHCAT to create pADHCAT89X5. The expression plasmid used has been described elsewhere³⁶. CAT activity was determined by the phase extraction method³⁵.

the expression of these genes and that additional levels of transcriptional or translational control contribute to the high levels of target gene expression seen in the most mature neuronal cells.

Activation

The initial yeast screen which identified Olf-1 was based on the ability of the cloned product, potentially fused to the GAL4 activator sequence, to stimulate transcription of *HIS3*. To assess the potential of Olf-1 to transactivate in a mammalian cell line, HEK 293 cells were cotransfected with Olf-1 cDNA and a reporter construct containing five Olf-1 sites upstream of a minimal promoter and the bacterial chloramphenicol acetyl transferase (CAT) gene. Cotransfection with the Olf-1 cDNA increased CAT enzyme activity more than 25-fold in an Olf-1 site-dependent fashion (Fig. 6). These data confirm that Olf-1 protein was sufficient to direct transcriptional activation in a mammalian cell line.

When extracts prepared from Olf-1 transfected cells are used in mobility shift assays, the complex detected is significantly slower in mobility than that detected in nuclear extracts prepared from olfactory tissue (data not shown). The mobility of the shifted product detected in the cell line is identical to that seen with *in vitro* translated Olf-1 protein. Proteolysis of the Olf-1 activity derived from tissue extracts may produce this difference in mobility. Although the Olf-1 protein that we have cloned demonstrates appropriate binding specificity and transcriptional activation as a homomultimer, we are currently examining whether heteromultimeric forms exist *in vivo*.

Discussion

We have successfully exploited a yeast genetic system to clone a specific transcription factor using its known target genes. In contrast to biochemical methods, selection in yeast does not require large amounts of starting material. Moreover, the expression of libraries in a eukaryotic system may allow for more efficient identification of candidate clones in comparison to bacterial expression libraries. Yeast represents a powerful

system for the genetic selection of candidate clones that may encode specific DNA-binding proteins and transcriptional activators.

The myogenic and achaete schute bHLH genes encode transcriptional activators which specify cell fate and require the helix-loop-helix for binding. We have demonstrated that the Olf-1 gene encodes an olfactory tissue-specific transcriptional activator containing a novel helix-loop-helix domain essential for DNA binding. Although the Olf-1 protein has the capacity to activate synthetic promoters in transformed cell lines, it remains to be shown whether Olf-1 expression is sufficient to induce expression of endogenous olfactory-specific genes. The data presented are consistent with Olf-1 functioning as a homodimer *in vivo* that is present as a proteolysed dimer in nuclear extracts. Other transcription factors, most notably NF κ B (ref. 26), undergo regulated proteolysis, which activates function.

The organization of sequences required for oligomerization and DNA binding distinguish Olf-1 from the previously known HLH transcription regulators and suggests that the bHLH family of proteins may also have unrecognized binding activities mediated by residues outside of the basic domain. The rHLH of Olf-1 may be a conserved structural motif present in other transcription factors; low-stringency hybridization of genomic Southern blots with a small fragment containing this region of the Olf-1 cDNA reveals several bands. Related rHLH proteins could function in olfactory differentiation in a combinatorial fashion as seen with the myogenic HLH proteins and their partners. Alternatively, other members of the rHLH family may function during the differentiation of other neuronal tissues.

Note added in proof: Hagman *et al.* have described the purification and cloning of the transcription factor EBF³⁹ from an early B-cell line. This gene appears to be an alternatively spliced form of the rat Olf-1 factor described here. The relationship between Olf-1 and EBF, the role of possible heterodimerization, post-translational modification and splice variants, and the nature of the respective binding sites in target genes will be subjects of future interest. □

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1. Reed, R. R. *Neuron* **8**, 205–209 (1992).
2. Graziadei, P. P. C. & Monti Graziadei, G. A. *The Olfactory System: A Model for the Study of Neurogenesis and Axon Regeneration in Mammals* 131–153 (Raven, New York, 1978).
3. Jones, D. T. & Reed, R. R. *Science* **244**, 790–795 (1989).
4. Bakalyar, H. A. & Reed, R. R. *Science* **250**, 1403–1406 (1990).
5. Dhallan, R. S., Yau, K. W., Schrader, K. A. & Reed, R. R. *Cell* **347**, 184–187 (1990).
6. Ludwig, J., Margalit, T., Eismann, E., Lancet, D. & Kaupp, B. *FEBS Lett.* **270**, 24–29 (1990).
7. Rodgers, K. E. *et al. Proc. Natn. Acad. Sci. U.S.A.* **84**, 1704–1708 (1987).
8. Davis, R. L., Weintraub, H. & Lassar, A. B. *Cell* **51**, 987–1000 (1987).
9. Lassar, A. B. *et al. Cell* **58**, 823–831 (1989).
10. Weintraub, H. *et al. Science* **251**, 761–766 (1991).
11. Vinson, C. R., LaMarco, K. L., Johnson, P. F., Landschultz, W. H. & McKnight, S. L. *Genes Dev.* **2**, 801–806 (1988).
12. Singh, H., LeBowitz, J. H., Baldwin, J. A. S. & Sharp, P. A. *Cell* **52**, 415–423 (1988).
13. Gruenberg, D., Natesan, S., Alexandre, C. & Gilman, M. *Science* **257**, 1089–1095 (1992).
14. Fields, S. & Song, O.-K. *Nature* **340**, 245–246 (1989).
15. Wilson, T. E., Fahrner, T. J., Johnston, M. & Milbrandt, J. *Science* **252**, 1296–1300 (1991).
16. Kozak, M. *Nucleic Acids Res.* **12**, 857–872 (1984).
17. Henthorn, P., Kiledjian, M. & Kadesch, T. *Science* **247**, 467–470 (1990).
18. Blackwood, E. M. & Eisenman, R. N. *Science* **251**, 1211–1217 (1991).
19. Benzeira, R., Davis, R. L., Lockshon, D., Turner, D. L. & Weintraub, H. *Cell* **61**, 49–59 (1990).
20. Anthony-Cahill, S. J. *et al. Science* **255**, 979–983 (1992).
21. Voronova, A. & Baltimore, D. *Proc. Natn. Acad. Sci. U.S.A.* **87**, 4722–4726 (1990).
22. Davis, R. L., Cheng, P.-F., Lassar, A. B. & Weintraub, H. *Cell* **60**, 733–746 (1990).

23. Tapscott, S. J. *et al. Science* **242**, 405–411 (1988).
24. Monti-Graziadei, P. P. C. in *Neural Growth and Differentiation* (eds Meisami, E. & Brazier, M. A. B.) 373–396 (Raven, New York, 1979).
25. Verhaagen, J., Oestreicher, W. H., Gispen, W. H. & Margolis, F. L. *J. Neurosci.* **9**, 683–691 (1989).
26. Fan, C. M. & Maniatis, T. *Nature* **354**, 395–398 (1991).
27. Chevray, P. M. & Nathans, D. *Proc. Natn. Acad. Sci. U.S.A.* **89**, 5789–5793 (1992).
28. Sikorski, R. S. & Heiter, P. *Genetics* **122**, 19–27 (1989).
29. Gietz, D., St. Jean, A., Woods, R. A. & Schiestl, R. H. *Nucleic Acids Res.* **20**, 1425–1425 (1992).
30. Chou, P. Y. & Fasman, G. D. *Rev. Biochem.* **47**, 251–276 (1978).
31. Garnier, J., Osguthorpe, D. J. & Robson, B. J. *molec. Biol.* **120**, 97–120 (1978).
32. Levy, N. S., Bakalyar, H. A. & Reed, R. R. *J. Ster. Biochem. molec. Biol.* **39**, 633–637 (1991).
33. Dignam, J. D., Lebowitz, R. M. & Roeder, R. G. *Nucleic Acids Res.* **11**, 1475–1489 (1983).
34. Smith, D. B. & Johnson, K. S. *Gene* **67**, 31–40 (1988).
35. Ausubel, F. M. *et al. Current Protocols in Molecular Biology* (Wiley, New York, 1990).
36. Gorman, C. M., Gies, D. R. & McCray, G. *DNA and Protein Engineering Techniques* **2**, 3–10 (1990).
37. England, B. P., Ulrike, H. & Tjian, R. *J. biol. Chem.* **265**, 5086–5094 (1990).
38. Kudrycki, K. *et al. Molec. cell. Biol.* **13**, 3002–3014 (1993).
39. Hagman, J. *et al. Genes Dev.* **7**, 760–763 (1993).

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The X-ray-emitting trail of the nearby pulsar PSR1929 + 10

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PULSARS—rapidly spinning, highly magnetized neutron stars—are thought to lose their rotational energy through the emission of relativistic particles^{1,2}. As the typical lifetime of a pulsar greatly exceeds that of the remnant of the supernova explosion in which it was born, the relativistic wind from older pulsars should interact directly with the interstellar medium; this can give rise to observable pulsar-wind nebulae³. Here we report the detection by the X-ray satellite *Rosat* of a different type of nebula, associated with the nearby pulsar PSR1929 + 10. The nebula appears as a linear diffuse X-ray feature in the direction opposite to the pulsar's proper motion. In this case, the pulsar wind appears to be confined by the ram-pressure arising from the high velocity of the pulsar through the interstellar medium, resulting in a trail of relativistic electrons with enhanced emissions of synchrotron radiation. Many such nebulae may exist in the solar neighbourhood, but they will be difficult to detect except in those relatively rare cases where the trails point nearly towards us.

PSR1929 + 10 is one of the nearest radio pulsars, with a rotational period of 0.227 s and a spin-down age of $10^{6.5}$ yr. The pulsar, located near the galactic plane ($l = 47.4$; $b = -3.9$), has a very small dispersion measure of $3.2 \text{ cm}^{-3} \text{ pc}$ and a line-of-sight H I column density of $1.4 \times 10^{20} \text{ cm}^{-2}$ (ref. 4). Although two available parallax measurements disagree in their estimates of the pulsar's distance ($45^{+27}_{-13} \text{ pc}$ according to ref. 5; $\sim 250 \text{ pc}$ according to ref. 6), a distance of 170 pc is consistent with the pulsar's dispersion measure⁷. At this distance, the pulsar's proper motion measured by Lyne *et al.* (ref. 8) corresponds to a transverse velocity of 70 km s^{-1} . The ram-pressure associated with the pulsar's motion would be $p_r = 2.3 \times 10^{-10} n_H v_{100}^2 \text{ dyn cm}^{-2}$, where n_H is hydrogen number density of the interstellar medium (ISM) (assuming solar abundance) and v_{100} is the total pulsar speed in units of 100 km s^{-1} . When this pressure exceeds the total ISM pressure, p_a , which is about $3 \times 10^{-12} \text{ dyn cm}^{-2}$ (ref. 9), the ram-pressure is expected to confine the relativistic wind from the pulsar, producing a spatially extended synchrotron emission source which is offset from the pulsar. This emission provides a natural explanation for the X-ray trail detected in the *ROSAT* image.

We discovered the trail by chance during the current archival study of the galactic soft X-ray background with deep images taken with the positive-sensitive proportional counter (PSPC) on board the *ROSAT* X-ray Observatory. For each observation, we first subtracted discrete sources detected with signal-to-noise ratios larger than 4 (equivalent to energy fluxes $\geq 10^{-14} \text{ erg s}^{-1} \text{ cm}^{-2}$ in the 0.5–2 keV band), and we then studied the diffuse background by calculating the autocorrelation function of observed diffuse X-rays. In the deep ($4 \times 10^4 \text{ s}$) observation of PSR1929 + 10, originally proposed by D. Helfand, a positive correlation was first detected on scales much greater than the size ($\sim 30''$ full width half maximum (FWHM)) of the point spread function of the instrument, indicating the presence of diffuse X-ray emission structure in the field. Images constructed in different energy bands were then examined. In the 0.5–2 keV band, a linear diffuse feature (or an emission trail) can be identified, which starts at the location of the pulsar and extends to the southwest for about $10'$ (Fig. 1). This feature nearly vanishes in the 0.1–0.3 keV band, consistent with the expected absorption along the path to the pulsar. The feature appears in the direction opposite to the pulsar's proper motion⁸,

as first pointed out by Helfand (personal communication). This strongly indicates that the feature is a result of the motion of the pulsar relative to the ISM.

To assess the significance of the trail, we have calculated the surface brightness distribution of diffuse X-rays as a function of the angular position around the pulsar. Discrete sources as marked in Fig. 1 are subtracted by removing around individual sources circular regions of twice the 90% radii (defined to contain 90% of the expected counts for point-like sources). In doing so, we may have excluded sources which are actually emission peaks of the trail, particularly in large off-axis regions where source confusion becomes serious because of the reduced spatial resolution and sensitivity of the instrument. Thus, our calculation is confined to the very central region ($\leq 12.5'$) of the image. In the angle interval 110 – 130° (west of north) along the direction of the trail (Fig. 1), the calculated distribution shows a clear enhancement. This enhancement has an integrated count rate of $(1.1 \pm 0.2) \times 10^{-2} \text{ counts s}^{-1}$ over a region of 21.6 arcmin^2 and a signal-to-background noise ratio of 5.3. The background noise is estimated from the surface brightness variation at angles away from the enhancement. The probability that random background noise produces the enhancement at any angular position in the image is only $\sim 10^{-4}$. It is also unlikely that the enhancement, with its count rate equivalent to about 10 sources just below our detection limit, represents a lining-up of undetected discrete sources. In particular, we can rule out the possibility that extragalactic sources (such as active galactic nuclei) could significantly contribute to the enhancement, because the large galactic absorption ($\sim 5 \times 10^{21} \text{ cm}^{-2}$) along the line of sight makes the spectrum of the contribution much too hard to be comparable to the observation.

To estimate the spectral shape of the X-ray emission from the trail, we have calculated count rates in three PSPC bands: 0.2–0.5 keV, 0.5–0.9 keV and 0.9–2 keV. The obtained count rates, $(3.2 \pm 1.6) \times 10^{-3}$, $(4.0 \pm 1.4) \times 10^{-3}$ and $(6.8 \pm 1.3) \times 10^{-3} \text{ counts s}^{-1}$, compared with an assumed power-law spectrum with the absorption along the path to the pulsar, give an energy slope $\alpha = 0.5^{+0.3}_{-0.4}$, a typical value for synchrotron emission sources such as the Crab nebula. Given the spectrum, we find the energy flux of the trail to be $(3.7 \pm 0.8) \times 10^{-13} \text{ erg s}^{-1} \text{ cm}^{-2}$ in the 0.5–2 keV band (the error bar includes the uncertainty in the spectral shape). For a pulsar distance of 170 pc, the corresponding luminosity is $(1.3 \pm 0.3) \times 10^{30} \text{ erg s}^{-1}$, or 3×10^{-4} of the pulsar's spindown luminosity.

A single dish radio survey at 11 cm (with a beam size $\sim 5'$) has placed an upper limit 66 mJy per beam on the surface brightness of the region nearby the pulsar¹⁰, or $\sim 0.2 \text{ Jy}$ on the total radio flux of the trail. Together with the trail's X-ray flux, this suggests that the power-law spectral slope must be flatter than 0.8, consistent with the trail's X-ray spectrum.

We find that the observed X-ray emitting trail can be explained naturally as synchrotron radiation from the ram pressure-confined wind of the pulsar PSR1929 + 10. As was postulated for the Crab pulsar^{1,2}, a pulsar loses its rotational energy primarily through an ultra-relativistic wind that consists of electron-positron pairs and magnetic field. When the pulsar is young, the wind interacts with the remnant of the supernova explosion from which the pulsar was born^{11–13}. But as the typical lifetime of a pulsar ($\sim 10^7 \text{ yr}$) is much longer than that of a remnant (a few times 10^5 yr), the pulsar will eventually be interacting with the general ISM. Blandford *et al.*³ argued that, in the case of a slowly moving pulsar, the relativistic electrons in the pulsar wind radiate in the interstellar magnetic field, which may be observable. Here, we propose a different type of pulsar wind nebulae associated with fast-moving pulsars. The surface brightness of such a nebula can be greatly enhanced if the relativistic electrons and magnetic fields from a pulsar are confined by the high ram-pressure due to the motion of the pulsar through the ISM.

As PSR1929 + 10 is expected to be interacting with the general ISM and is most probably moving supersonically relative to its

ambient medium, a bow shock is expected to run ahead of the pulsar to sweep up the ambient medium, and a reverse shock should terminate the freely streaming pulsar wind. Although the length scale $l_s \approx 7 \times 10^{15} n_H^{-1/2} v_{100}^{-1}$ cm (ref. 14) characterizes the distance of the reverse shock from the pulsar, the approximate distance to the contact discontinuity from the pulsar $l_c \approx 1.5 l_s$ (ref. 15), or $l_c \approx 4'' n_H^{-1/2} v_{100}^{-1}$ may be used to characterize the size of the synchrotron emission region close to the pulsar. Apparently, this region is not resolved in the PSPC image of the pulsar.

The shocked pulsar wind materials, once they emerge from the reverse shock, are blown behind the pulsar by the ram-pressure, and flow inside a tunnel in a direction opposite to that of the pulsar motion. As the pressure along the tunnel is expected to decrease rapidly over distance of order a few times l_c , a de Laval nozzle is expected to form¹⁶, inside which the relativistically hot shocked pulsar wind materials will be accelerated quickly to relativistic, supersonic speed by the pressure gradient. After the flow becomes supersonic, the tunnel keeps widening to lower the pressure of the flow, and eventually becomes a cylindrical tube at a distance on the order of $10^{17} n_H^{1/4} v_{100}^{1/2}$ cm, where the flow comes into pressure balance with the ambient medium. Assuming that the magnetic pressure is the same as the gas pressure, we can use the relativistic Bernoulli equation, together with the conservation of particle number and the equation of state for a relativistically hot fluid¹⁶, to derive the final radius of the tube $r \approx (p_r/p_a)^{1/4} l_s \approx 8'' n_H^{-1/4} v_{100}^{-1/2} p_0^{-1/4}$ (where p_0 is the ambient medium pressure in units of 3×10^{-12} dyn cm⁻²), with corresponding Lorentz factor of the bulk flow $\gamma \approx [17.5 v_{100} \sqrt{n_H/p_0 - 1}]^{1/2}$. This tube most probably represents the observed X-ray trail.

To come into equilibrium with its surroundings, the supersonic flow inside the tunnel must be terminated by a shock (or a series of oblique shocks) where most of the pulsar spin-down luminosity is dumped. The shock is expected to be located far behind the pulsar and is probably outside the region covered by Fig. 1.

We now examine whether the synchrotron radiation of the relativistic electrons inside the tunnel can explain the observed X-ray luminosity of the trail. Adopting a formula for the synchrotron emissivity from ref. 17 and assuming a power-law energy distribution function of the relativistic electrons with an index of 2 (or an emission spectral slope of $\alpha = 0.5$), we find that the integrated 0.5–2 keV luminosity of the tunnel can be expressed as

$$L_{x,t} \approx 2.6 \times 10^{28} \frac{p_0^{5/4} l_{18}}{n_H^{1/2} v_{100} \ln(\varepsilon_2/\varepsilon_1)} \left[\frac{1}{\gamma(1 - \beta \cos \theta)} \right]^{2.5} \text{ erg s}^{-1} \quad (1)$$

where l_{18} is the total length of the tunnel in units 10^{18} cm, and ε_1 and ε_2 represent the lower and upper energy limits of the electrons. The term in square brackets represents the Doppler boosting of the synchrotron radiation¹⁸, and we have denoted the dimensionless speed of the flow by β and the angle between the line of sight and the tunnel axis by θ . At a distance of 170 pc, if we assume $p_0 = 1$ and take a moderate pulsar speed of 300 km s^{-1} (ref. 7) and $n_H = 10^{-2} \text{ cm}^{-3}$ (appropriate for the ISM coronal component which may dominate the region near the pulsar¹⁹), then we have $\theta = 13.5^\circ$ (since the proper motion of the pulsar is 70 km s^{-1}). This yields $l_{18} = 7.3$ and $\gamma = 2.1$ (thus $\beta = 0.87$). According to equation (1), we then have $L_{x,t} \approx 1.2 \times 10^{30} [10/\ln(\varepsilon_2/\varepsilon_1)] \text{ erg s}^{-1}$. The quantity inside the square brackets should be close to unity, if the value of $\varepsilon_2/\varepsilon_1$ does not differ much from that found in the magnetohydrodynamics model of the Crab nebula².

It should be pointed out that the gyration radii of the X-ray emitting electrons are comparable to the width of the tube, and the diffusion of some of these electrons outside the tube may affect our estimate. Instabilities along the wall of the tube may

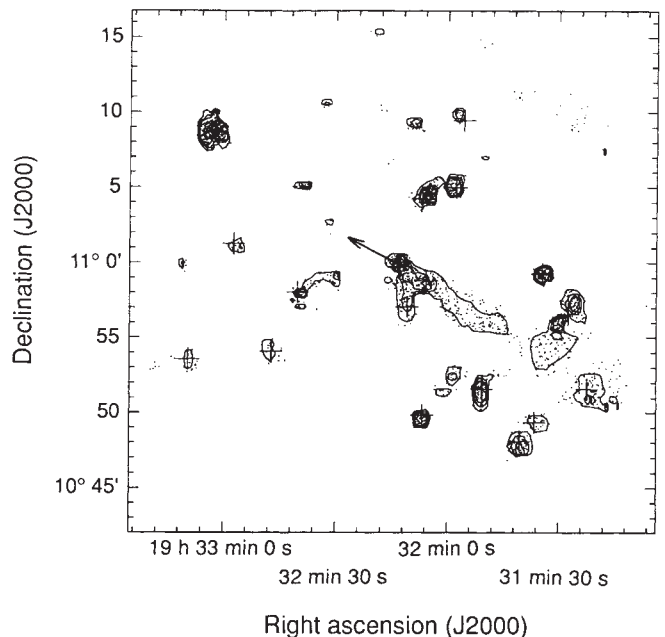


FIG. 1 The surface brightness of the X-ray emission in the field with the pulsar PSR1929+10 at the centre. North is up and east to the left. The surface brightness has been calculated from data collected in the 0.5–2 keV band within the central region (within $17.5'$ of the axis; outside this the quality of the data degrades considerably) of the PSPC observation and has been smoothed with an adaptive filter (with apertures $\leq 2'$) to display the faint diffuse feature together with bright discrete sources. The logarithmic contour levels in units of counts $\text{s}^{-1} \text{ deg}^{-2}$ are 0.4, 0.5, 0.6, 1.0, 1.5 and 2, respectively. The locations of discrete X-ray sources are marked by plus signs (+). The arrow represents the direction of the pulsar's proper motion ($63^\circ \pm 3^\circ$ (J2000) east of north; ref. 5).

lead to entrainment and mixing, which would slow the flow (and thus increase the synchrotron emissivity) in a boundary layer²⁰. Although the above combination of parameters is not unique, our simple model reproduces the observed X-ray luminosity of the trail within its uncertainties. To explain the observed X-ray luminosity, relativistic beaming effects (contributing a factor of 19 for the above parameter combination) must be important unless either the pressure inside the X-ray emitting region is much higher than the ambient medium pressure that we adopted (by a factor of ~ 10), or the width of this region is much larger than the width of the tube estimated here ($\sim 30''$), or both.

Similarly, we may estimate the X-ray emission of the shocked pulsar wind in the bow shock region (see also ref. 21) and in the region connecting the bow shock region and the tube. We find that this emission could account for at least a part of the unpulsed X-ray luminosity $\sim 4 \times 10^{29} \text{ erg s}^{-1}$ (at a distance of 170 pc) estimated from the same PSPC observation²².

Follow-up radio, optical and X-ray observations should provide further information on the trail's emission morphology and spectrum, useful for detailed modelling of the interactions of the pulsar wind with the ISM, and the formation and structure of the pulsar wind nebula. Furthermore, one could search for nearby unknown pulsars by detecting the radiation from the pulsar trails. But if the radiation does come from relativistic, supersonic flows inside narrow tunnels, it may be difficult to detect except in the rare cases where the tunnels point nearly towards us. $\square$

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1. Rees, M. & Gunn, J. *Mon. Not. R. Astr. Soc.* **167**, 1–12 (1974).
2. Kennel, C. F. & Coroniti, F. C. *Astrophys. J.* **283**, 694–709 (1984).
3. Blandford, R. D. et al. *Astr. Astrophys.* **23**, 145–146 (1973).
4. Weisberg, J. M., Rankin, J. M. & Borjesson, V. *Astr. Astrophys.* **88**, 84 (1980).

5. Salter, M. J., Lynne, A. G. & Anderson, B. *Nature* **280**, 477–478 (1979).
6. Backer, D. C. & Sramek, R. A. *Astrophys. J.* **260**, 512–519 (1982).
7. Taylor, J. H., Manchester, R. N. & Lyne, A. G. Princeton preprint (1993).
8. Lyne, A. G. & Anderson, B. & Salter, M. J. *Mon. Not. R. Astr. Soc.* **201**, 503–520 (1982).
9. Cox, D. P. in *The Interstellar Medium in Galaxies* ed. Thronson, H. A. Jr & Shull, J. M. 181–200 (Dordrecht, Kluwer, 1989).
10. Velusamy, T. & Kundu, M. R. *Astrophys. Lett.* **17**, 177–182 (1976).
11. Li, Z.-Y. & Begelman, M. C. *Astrophys. J.* **400**, 186–191 (1992).
12. Frail, D. A. & Kulkarni, S. R. *Nature* **352**, 785–787 (1991).
13. Shull, J. M., Fesen, R. A. & Saken, J. M. *Astrophys. J.* **466**, 860–868 (1989).
14. Weaver, R. et al. *Astrophys. J.* **218**, 377–395 (1977).
15. Van Buren, D. & McCray, R. *Astrophys. J.* **329**, 93–96 (1988).
16. Landau, L. D. & Lifshitz, E. M. *Fluid Mechanics* (Pergamon, London, 1959).
17. Rybicki, G. B. & Lightman, A. P. *Radiative Processes in Astrophysics*, 180 (Wiley, New York, 1979).
18. Begelman, M. C., Blandford, R. D. & Rees, M. J. *Rev. mod. Phys.* **56**, 255–352 (1984).
19. Paresce, F. *Astrophys. J.* **89**, 1022 (1984).
20. Kulkarni, S. R., Phinney, E. S., Evans, C. R. & Hasinger, G. R. *Nature* **359**, 300–302 (1992).
21. Cheng, A. F. *Astrophys. J.* **275**, 790–801 (1983).
22. Yancopoulos, S. et al. *Bull. Am. astr. Soc.* **24**, 1217 (1993).

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Flux pinning, granularity and the irreversibility line of the high- T_c superconductor $\text{HgBa}_2\text{CuO}_{4+x}$

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THE recent discoveries of superconductivity at 94 K in the single- CuO_2 -layer compound $\text{HgBa}_2\text{CuO}_{4+x}$ (Hg-1201)¹ and at 133 K in its two- and three-copper-layer analogues² (Hg-1212 and Hg-1223) have renewed interest in the search for new high-transition-temperature (high- T_c) superconductors. But whatever the T_c , high values of intergrain critical current density are required for large-scale applications; thus, the material must have good electromagnetic connectivity between grains (it must not be electromagnetically granular) and it should be able to maintain its current-carrying capacity in high magnetic fields (it should have a high irreversibility field, $H^*(T)$). Putilin *et al.*¹ surmised that the small CuO_2 layer spacing of Hg-1201 might yield a high $H^*(T)$. We report here that $H^*(T)$ is indeed significantly higher than for the two- and three-copper-layer Bi-Sr-Ca-Cu-O compounds (Bi-2212 and Bi-2223), but lower than for $\text{YBa}_2\text{Cu}_3\text{O}_7$ (Y-123). The low-temperature flux pinning is also strong. Like Y-123, however, Hg-1201 is electromagnetically granular. A high degree of grain alignment will probably be necessary to remove this granularity; by analogy with the bismuth compounds, this is likely to be easier if the two-mercury-layer counterparts of Bi-22XY (Hg-2201, -2212 or -2223) can be synthesized.

$\text{HgBa}_2\text{CuO}_{4+x}$ was prepared from stoichiometric mixtures of HgO and Ba_2CuO_3 . Ba_2CuO_3 was prepared by reacting BaO_2 (<95%) and CuO (<99.9%) at 900°C in oxygen for 24 hours using one intermediate grinding step. Storage, grinding, and mixing of Ba_2CuO_3 with HgO was done in an inert atmosphere. Pellets of the mixture were placed in an alumina crucible, which in turn was placed inside a quartz tube. This was evacuated, sealed, then placed in a stainless steel tube filled with sand to protect against possible explosion (which did not occur). The samples studied here were heated to 800 °C at 5 °C min⁻¹, then slowly cooled to room temperature over 10 hours. Samples heated at 845 °C exhibited sharper X-ray patterns for the 1201 phase but also showed free Hg, as also seen by Wagner *et al.*³

For this reason we worked at 800 °C. Subsequent transmission electron microscopy (TEM) on the 800 °C samples showed that they too had some free Hg (or HgO) within them, the size of these particles being 10–30 nm. Our X-ray, scanning electron microscopy (SEM), TEM, and magnetization measurements showed that the proportion of 1201 phase was > 50%. After reaction, the samples were annealed at 200 °C for 2–6 hours in flowing oxygen. The grain size of all samples was ~ 5 µm and the grain shape was nearly equiaxed. Mercury-rich compounds were observed at a number of grain boundaries, though others appeared clean, when examined by back scatter SEM and TEM.

The essence of our results is shown in Figs 1–4. Figure 1a shows the a.c. susceptibility transition of the as-reacted and subsequently oxygenated samples in a zero-to-peak field of 0.1 mT. Both transitions are rather more abrupt than in the original report¹. The treatment in oxygen markedly raised the T_c onset from ~ 80 K to ~ 91 K. Both samples appeared electromagnetically well-connected under this small a.c. field and the sharp transition indicates the basic good quality of the Hg-1201 phase. Raising the a.c. field however, from 0.1 to 4 mT, produced a marked broadening of the transition (shown in Fig. 1b) consistent with electromagnetic granularity caused by the field breaking the intergrain shielding current loops. The d.c. magnetization was measured in a vibrating sample magnetometer (VSM) at fields up to 12 T from 4 to 80 K. The critical current density (J_{cm}) was calculated from the modified Bean expression⁴ for the magnetization hysteresis $\Delta M = J_{cm}a_2(1 - (a_2/3a_1))/10$, where $2a_1$, $2a_2$ are the cross sectional dimensions and $a_1 > a_2$. The whole sample cross-section (1.5 mm × 2 mm) was used to derive J_{cm} in the limit that all the current was flowing from grain to grain (intergrain). The grain size (~ 5 µm) was

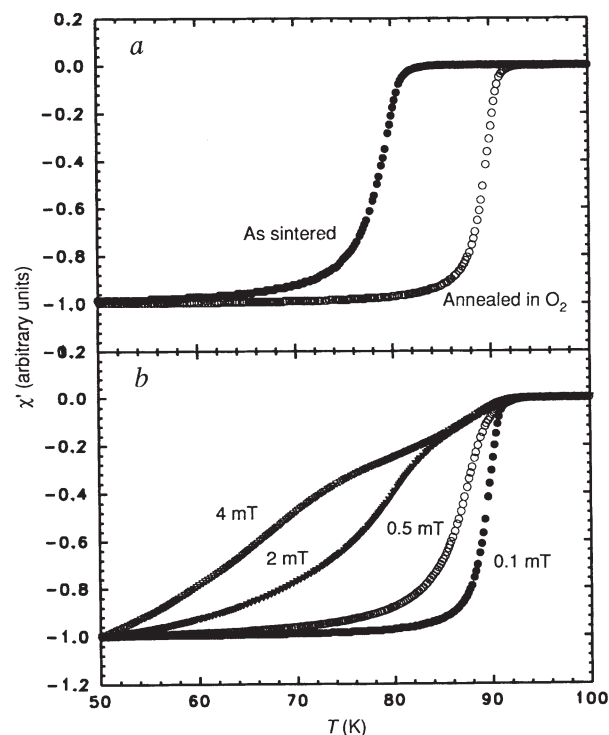


FIG. 1 a, Real component of the susceptibility (χ') for the as-sintered and oxygen annealed samples at $H=0.1$ mT. b, $\chi'(T)$ of the oxygen annealed sample for a.c. fields between 0.1 and 4 mT.

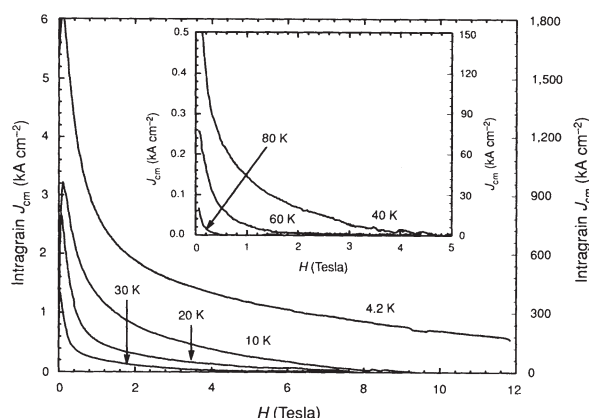


FIG. 2 Magnetization critical current densities (J_{cm}) for fields up to 12 T from 10 to 80 K. The right-hand vertical scale represents the intragrain J_{cm} while the left-hand scale represents the intergrain J_{cm} .

used to determine J_{cm} for the limit of current flow only within individual grains (intragrain). Figure 2 presents these data at temperatures from 4 to 80 K. Using the intergrain calculation, the J_{cm} (4 K) values reach only a low maximum value of $\sim 6 \times 10^3 \text{ A cm}^{-2}$, while they become significantly larger ($\sim 2 \times 10^6 \text{ A cm}^{-2}$) in the intragrain limit. The temperature dependence of J_{cm} was found to be quite large and both the magnetization hysteresis and J_{cm} were observed to fall to zero at the irreversibility field $H^*(T)$ at all temperatures above 10 K. Figure 3 plots $H^*(T)$ against the normalized temperature T/T_c and compares it to published⁵ irreversibility lines for Y-123, Bi-2212 and Bi-2223. It also includes just received data on a sample of Hg-1201 measured by Welp *et al.*⁶. The normalized behaviour of $H^*(T)$ for Hg-1201 is rather close to that of the La-214 compound; however, the absolute behaviour is much better, because the T_c of Hg-1201 is more than twice as high. Y-123, having an almost identical T_c but a larger interplanar spacing, has a much higher irreversibility line, but the lines for Bi-2212 and Bi-2223 are lower than for Hg-1201. The agreement between our swept-field

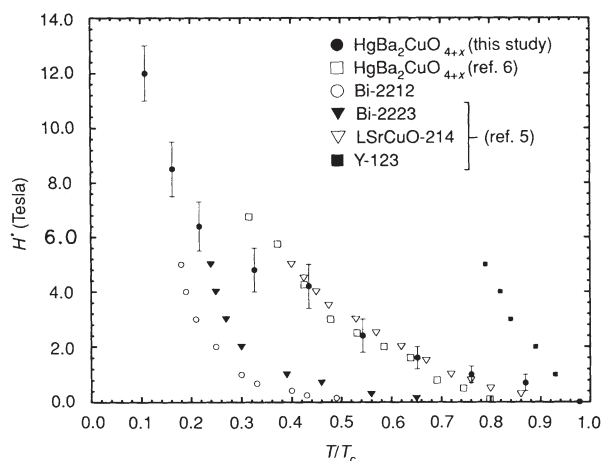


FIG. 3 Irreversibility field (H^*) plotted against reduced temperature (T/T_c). $H^*(T)$ for Bi-2212, Bi-2223, LaSrCuO-214, Y-123 (ref. 5) and HgBa₂CuO_{4+x} (ref. 6) are plotted for comparison.

magnetization data and the SQUID measurements of Welp *et al.* on different Hg-1201 samples is good and suggests that these values for $H^*(T)$ are only weakly dependent on the voltage criterion.

The flux pinning behaviour of the compound can be inferred from Fig. 4, which plots the pinning force $F_p (= |J_{cm} \times B|)$ against magnetic field H . By normalizing F_p and H to their maximum values $F_{pmax}(T)$ and $H^*(T)$, respectively, we observe good scaling of $F_p(H)$ from 10 to 60 K. The curves superimpose reasonably well, and can be described by the formula $F_p \propto h^{1/2} (1-h)^\alpha$, where $\alpha = 1.15$ and $h \equiv H/H^*$. The normalization of H to H^* accounts phenomenologically for the strong flux creep in high- T_c superconductors for which H^* can be well below the upper critical field H_{c2} . Similar scaling, albeit with a different shape of $F_p(H)$ has been observed for Y-123 (ref. 7) and Bi-2223 (ref. 8) epitaxial thin films exhibiting high transport J_c values and no granularity. The universality of the $F_p(H)$ curves at different temperature implies that either the sample is always connected, or it is always granular for the whole temperature and field range of Fig. 4. Granular behaviour is clear in the low-field a.c. susceptibility measurements (Fig. 1b). An additional check for granularity was made by comparing ΔM to the change of applied field ΔH necessary to invert the critical state. In the Bean model this ratio of $\Delta H/3\Delta M$ should be ~ 1 if the sample is coupled. At 4 K and 12 T, this value was in fact 21, a number characteristic of a strongly granular material⁹. We thus conclude that the J_{cm} and F_p data derived from the magnetization measurements should be entirely associated with the intragrain critical current density.

The first requirement for large-scale applications of any high- T_c superconductor (HTS) is that the material be available in polycrystalline form which is not electromagnetically granular. This is precisely the reason why Y-123 has found no useful bulk application. Unfortunately, Hg-1201 appears similar to Y-123 in this respect, being electromagnetically granular in all but very weak fields. Of course, a better understood and more controlled synthesis procedure may yield non-granular material, although intense efforts over 6 years have not yet succeeded in this for Y-123. Prospects for bulk applications of HTSs presently depend on Bi-2212 and Bi-2223. The easily broken double Bi-O layer of these compounds encourages a plate-like, aligned and electromagnetically connected grain morphology having high grain-to-grain J_c values. This suggests that the synthesis of the double Hg-O layer analogues of Hg-12XY should be a high

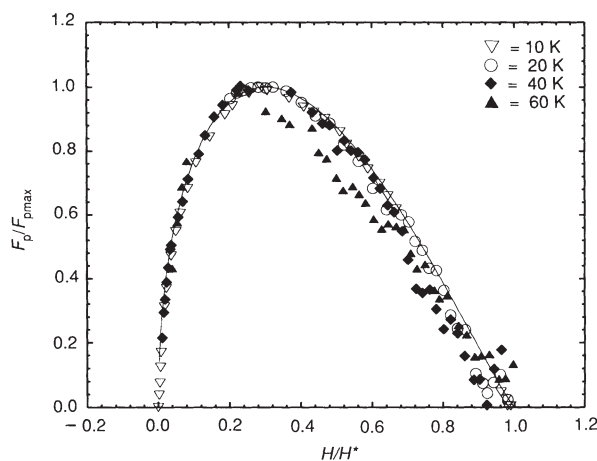


FIG. 4 Normalized pinning force $F_p(H)/F_{pmax}$ plotted against the reduced field $h = H/H^*$ at 10, 20, 40 and 60 K. The solid line is described by the $F_p/F_{pmax} \propto h^{1/2}(1-h)^{1.15}$ expression.

priority; by analogy to the one and two TI-O layer prototypes¹⁰, the T_c values of Hg-22XY should also be higher than the present 133 K record² for Hg-1223. The second requirement for applications is that the flux pinning be strong; this is indeed the case for Hg-1201 in the low temperature limit and $H^*(T)$ is also higher than for Bi-2212 and Bi-2223, consistent with the conjectures of Putlin *et al.*¹ The irreversibility line however, is somewhat lower than for Y-123. On balance, Hg-1201 does not appear to have any particular advantage over existing high- T_c superconductors. The prospect of large-scale applications of Hg-1212 and Hg-1223 awaits studies similar to this one. If these compounds too are granular, this will emphasize the desirability of synthesizing the double-mercury-layer compounds. Even if their normalized $H^*(T)$ behaviour (fig. 3) were to be no better than that of their double-bismuth-layer analogues, their expected higher T_c values should give them higher absolute values of $H^*(T)$. A 15–20 K increase in $H^*(T)$ could be of immense technological significance if the new materials were to retain the good intergrain connectivity of Bi-2212 and Bi-2223. □

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- Putlin, S. N., Antipov, E. V., Chmisse, O. & Marezio, M. *Nature* **362**, 226–228 (1993).
- Schilling, A., Cantoni, M., Guo, J. D. & Ott, H. R. *Nature* **363**, 56–58 (1993).
- Wagner, J. L. *et al. Physica C* **210**, 447–452 (1993).
- Campbell, A. M. & Evetts, J. E. *Adv Phys.* **21**, 199–429 (1972).
- Suenaga, M., Welch, D. O. & Budhani, R. *Supercond. Sci. Technol.* **5**, S1–9 (1992).
- Welp, U. *et al. Appl. Phys. Lett.* (in the press).
- Satchell, J. S. *et al. Nature* **334**, 331–332 (1988).
- Yamasaki, H. *et al. Phys. Rev. Lett.* **70**, 3331–3334 (1993).
- Küpfer, H., Keller, C., Gurevich, A., Salama, K. & Seivamanickam, V. in *Advances in Superconductivity III* (eds Kajimura, K. & Hayakawa, H.) 709–711 (Springer, Tokyo, 1991).
- Raveau, B., Michel, C., Hervieu, M. & Grouit, D. in *Crystal Chemistry of High T_c Superconducting Copper Oxides* 123–140 (Springer, Berlin, 1991).

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Nutrient and sediment retention in Andean raised-field agriculture

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RAISED-FIELD agriculture was widespread throughout Central and South America in prehispanic times^{1,2}. In this system of agriculture, crops are cultivated on a series of raised beds, which are separated from one another by deep, water-filled channels. In some regions, rehabilitation of the raised fields is now underway, largely because this practice leads to fertile soils, adequate water supply and protection from frost and therefore to substantially higher yields than more conventional methods^{3,4}. Here we report analyses of water quality in the channels alongside rehabilitated raised fields in the vicinity of Tiwanaku, on the Bolivian side of the Lake Titicaca basin (Fig. 1). We find that high concentrations of nitrate, available phosphate and turbidity decline significantly as the water flows through the raised-field channels. Water flowing through control sites shows no significant change.

Retention of nutrients and suspended sediments in the channels helps to maintain soil fertility and reduces pollution of downstream waters. Thus it seems there are environmental benefits in rehabilitating raised fields, which complement and help sustain the economic benefits demonstrated previously^{3,4}.

The location of our study is the vicinity of Tiwanaku on the Bolivian side of the Lake Titicaca basin (Fig. 1). Our study of nutrient and sediment fluxes from land to the lake is part of the MAB/UNESCO (Man and the Biosphere/United Nations Educational, Scientific and Cultural Organization) land-inland water ecotone program^{5,6}. We sampled along nine transects which allowed us to determine the effects of watershed ecotone type (short and simpler to long and complex) and type of land use (conventional versus raised field)⁷. The horizontal distance from the start of surface water flow to the lake shore was used to place transects in one of three categories: less than 1.5 km (short); 1.5 to 3.5 km (intermediate); greater than 3.5 km (long). With increasing length there is greater diversity and complexity in land cover, soil type, land use, terrain (hills, valleys, plains) and nearshore vegetation zones through which water passes and can thus be altered.

In this paper we compare two raised-field sites to two other sites with conventional land uses (Fig. 1). All these sites are located within long ecotones. This report focuses on how nutrients and suspended sediment concentrations are affected in raised-field sites compared to locations in other transects with conventional fields. Nutrients and vegetation were sampled at three or more locations along each transect. Concentrations of soluble available nitrogen (nitrate, ammonium), soluble reactive phosphorus (phosphate) and turbidity are compared at two locations within transects: the base of foothills and start of the broad plain adjacent to the lakeshore, and about 150 metres downstream in the plain. In raised-field transects these points are at the inflow and outflow of the canal system. In the other

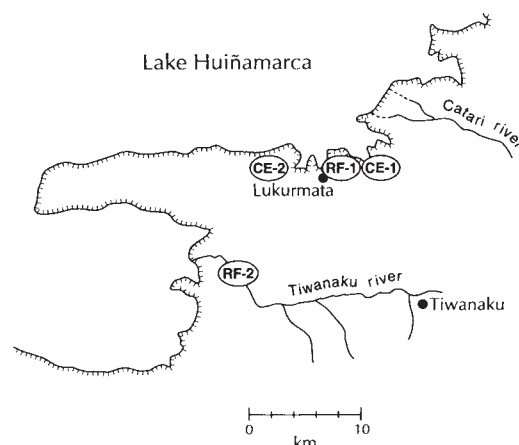
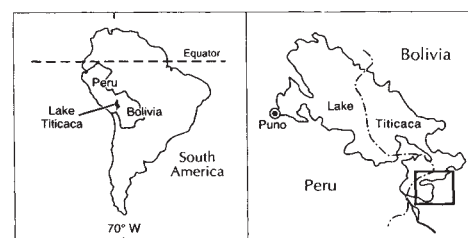


FIG. 1 Study region and transect sites in the Huiñamarca basin of Lake Titicaca. Codes for transects are: raised field 1-RF 1(Lukurmata); raised field 2-RF 2(Tiwanaku R.); conventional ecotone 1-CE 1(Chohasivi); conventional ecotone 2-CE 2(Huacullani).

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TABLE 1 Average nutrient inflow and outflow concentrations ($\mu\text{g l}^{-1}$ N or P $\pm$ standard error in parentheses) within ecotonal transects

Transect		Inflow	Outflow	O/I(%)
Raised field 1: (Lukurmata)				
	NO_3	4195(± 533)	57(± 20)	1.4
	PO_4	425(± 41)	34(± 6)	8
Raised field 2: (Tiwanaku R.)				
	NO_3	83(± 27)	43(± 19)	51.8
	PO_4	112(± 7)	35(± 5)	31.3
Conventional 1: (Chohasivi)				
	NO_3	3230(± 530)	464(± 108)	14.4
	PO_4	209(± 25)	64(± 8)	30.6
Conventional 2: (Huacullani)				
	NO_3	3338(± 364)	413(± 14)	12.4
	PO_4	234(± 38)	69(± 12)	29.5

Comparison of four transects from January 1990 to March 1991. Field sampling was monthly during the wet season and bimonthly during the dry season. Results of multiple-comparison tests¹⁶ between transects and other analyses are discussed in the text. The declines in nitrate and phosphate were significantly greater ($P < 0.05$) in the Lukurmata raised-field transect in comparison with the two conventional land use transects according to both the Fisher PLSD (protected least significant difference) and Scheffe *F*-tests. A one-factor repeated measures ANOVA (analysis of variance) showed significant differences in inflow minus outflow of both nutrients ($P = 0.013$ for NO_3 , $P = 0.017$ for PO_4) between these three transects. The Tiwanaku raised-field transect was not included in this analysis because the inflow values and variance there were much lower. Nitrate was measured with the cadmium reduction method¹⁷, and soluble reactive phosphorus was measured using the ascorbic acid/molybdenum blue method¹⁸.

transects these locations are along streams that flow within grasslands.

Nitrate is a nutrient of special interest because it can indicate land use changes which have important consequences for water quality^{8,9}. Nitrate moves readily through soil. Thus, the concentrations of this nutrient were quite high at the inflow to Lukurmata raised fields (average $4195 \mu\text{g l}^{-1}$ N Table 1) because the source was a groundwater spring. After water passed through the canals between raised fields, nitrate concentrations declined dramatically to a mean of $57 \mu\text{g l}^{-1}$ N at the outflow. The percent concentration in the outflow divided by inflow was substantially lower in this transect (1.4%) than in the other transects (Table 1). This may have been due to uptake of nitrate by the abundant macrophytes and algae in the raised-field canals. Experimental nutrient bioassays¹⁰ demonstrated that growth of the aquatic vegetation in the canals was limited by both nitrogen and phosphorus¹¹. This growth lowered concentrations of these nutrients in the canals. Other processes such as denitrification, nitrification and N mineralization in the canal sediments may also be important. This is the case in comparable systems of other regions¹².

Inflowing nitrate levels at the Tiwanaku raised-field site were relatively low ($83 \mu\text{g l}^{-1}$ N) because the source was the Tiwanaku River. Photosynthetic algae upstream in the river used and depleted this nutrient. Nitrate levels were reduced still further within this raised-field system to outflow levels ($43 \mu\text{g l}^{-1}$ N) that were below, but not significantly different from, the Lukurmata outflow values. In the two transects with conventional land use nitrate levels also declined substantially. Outflow levels (464 and $413 \mu\text{g l}^{-1}$ N), however, remained an order of

magnitude higher than at both raised-field sites (see below).

Soluble reactive phosphorus also declined substantially as the water passed through the raised fields. Inflow concentrations averaged $425 \mu\text{g l}^{-1}$ P at Lukurmata, with outflow concentrations declining to $34 \mu\text{g l}^{-1}$ P (Table 1). This was remarkably close to the outflow level of $35 \mu\text{g l}^{-1}$ P at Tiwanaku. In the long ecotones without raised fields, phosphorus concentrations also decreased, but only to 64 and $69 \mu\text{g l}^{-1}$ P. Multiple-comparison tests showed that declines in soluble reactive phosphorus concentrations were significantly greater in the Lukurmata raised-field transect ($P < 0.05$) than in the adjacent conventional long ecotones (Table 1). These results are similar to those for nitrate.

The transect near the Tiwanaku River demonstrates that raised fields can also filter out suspended sediments and thus dramatically increase water clarity (Table 2). Most raised fields we have observed, as in Lukurmata, are fed by small streams and springs with clear inflowing water. Fields in the Tiwanaku transect, however, are fed by very turbid water diverted from the Tiwanaku River. This turbidity is due to the high suspended sediment load in the river. At the inflow to these fields we have noted very high concentrations (mean 29.5 turbidity units). Within this raised-field system, the concentrations declined substantially to 5 turbidity units; at the outflow they were even lower (1.7 turbidity units). At Lukurmata the water was still less turbid at the outflow (0.9 units). We have not observed such dramatic reductions in turbidity in any other transects. In fact, in the conventional long ecotones, turbidity increased significantly to a mean value of 6.5 (Table 2).

Concentrations of nutrients and sediments flowing into the raised-field systems varied substantially according to whether the source was surface water or groundwater. Thus, in order to evaluate the retention capacity of raised-field systems we compared differences in outflow concentrations between pooled raised-field transects and pooled conventional long ecotone transects (Fig. 2). We found strikingly lower outflow concentrations in raised-field transects for both major nutrients and

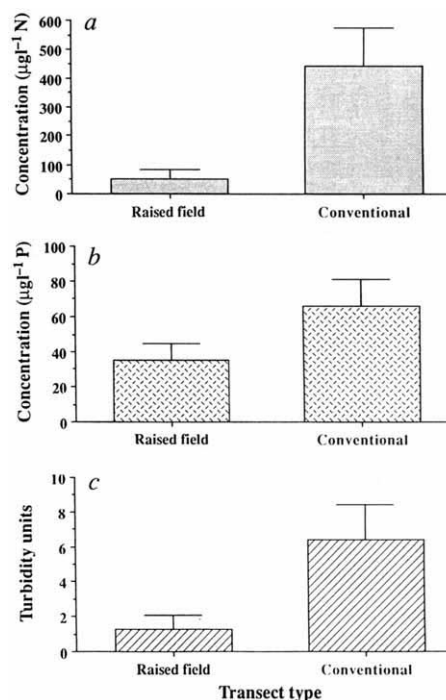


FIG. 2 Mean outflows of major nutrients and suspended sediments for raised fields and conventional land use in long ecotones (a nitrate, b phosphate, c turbidity). Error bars are 95% confidence limits.

TABLE 2 Average turbidity changes within ecotonal transects

Transect	Inflow	Outflow	O/I(%)
Raised Field 2	29.5(±4.0)	1.7(±0.5)	5.8
Raised Field 1	2.0(±1.1)	0.9(±0.2)	45
Conventional 1	2.0(±1.0)	5.0(±0.7)	250
Conventional 2	1.7(±0.6)	7.9(±1.2)	465

Turbidity was measured as absorbance spectrophotometrically¹⁹.

turbidity. This reflected enhanced and remarkably consistent retention of these materials by the two raised-field systems. The differences between raised fields and conventional methods were greatest for nitrate (Fig. 2a). The mean outflow at raised-field sites, 53 µg l⁻¹ N, was about one order of magnitude lower than at other long ecotones, 441 µg l⁻¹ N. Mean outflow concentrations of phosphate and suspended sediments were also significantly lower ($P < 0.05$) at raised-field sites.

In conclusion, this study indicates that there are environmental benefits of raised-field agriculture which help sustain improved yields. Nutrients are kept within the agricultural system since they are taken up in dissolved form by aquatic vegetation or are otherwise retained in canals. In addition, there is substantial growth in the canals of the water fern *Azolla* and its nitrogen-fixing endosymbionts. This vegetation is periodically recycled by farmers from canals to raised fields to serve as an organic fertilizer. We have observed extensive and regular use of two techniques for nutrient recovery during the rehabilitation: first, direct harvest of aquatic vegetation from the canals and second, shovelling of canal sediments into fields during the dry season, which both maintains the canal network and improves planting beds with organic nutrient-rich sediments. Archaeological evidence indicates that the farmers of Tiwanaku also used these techniques¹³. These practices add to the retention and recycling of essential nutrients and organic matter, and erosion is minimized. Thus, sediment and associated nutrients do not cause the downstream and nearshore pollution observed in other parts of the lake. Shifts in land use are recorded by clear changes in material fluxes to the nearshore sediments¹⁴. This nutrient flow and cycling in raised-field systems is in sharp contrast to regions where inorganic fertilizers are not retained in agricultural systems, and serious downstream nutrient contamination problems result^{8,9}. Macrophytes, which function like the raised-field canal vegetation, can mitigate such problems throughout the world¹⁵.

We should emphasize that the type and extent of these environmental benefits depends on the location of the raised fields in the landscape, hydrological conditions, and agricultural management practices. For example, the first raised-field site retains high levels of nutrients while turbidity is low throughout, whereas the second such site retains high suspended sediments while major nutrients are relatively low throughout. Ideally, raised fields should be located in the flat plains near the lake shore. This allows them to intercept and retain both nutrients and sediments from both nonpoint and major point sources before the water enters the lake. There is currently great variability in where and how raised fields are being rehabilitated. Thus, to help ensure more sustainable development, the environmental factors presented here should be considered and their applications optimized. A guiding principle should be the maintenance of the desirable features of wetlands: high productivity and biodiversity coupled with retention of soils and nutrients. As the raised-field agricultural expansion continues, long-term experimental research and monitoring of reconstructed raised fields are needed to further improve both economic and environmental benefits. □

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1. Denevan, W. M. *Science* **169**, 647–654 (1970).
2. Erickson, C. L. *Expedition* **30**(3), 8–16 (1988).
3. Kolata, A. L. & Ortloff, C. J. *Arch. Sci.* **16**, 233–263 (1989).
4. Bray, W. *Nature* **345**, 385 (1990).
5. Naiman, R. J., Decamps, H. & Fournier, F. (eds) *The Role of Land/Inland Water Ecotones in Landscape Management and Restoration: A Proposal for Collaborative Research*. 93 (MAB Digest 4) (UNESCO, Paris, 1989).
6. Naiman, R. J. & Decamps, H. (eds) *The Ecology and Management of Aquatic-Terrestrial Ecotones* 316 (Vol. 4, MAB/UNESCO Series) (Parthenon, London, 1990).
7. Carney, H. J., Binford, M. W., Marin, R. R. & Goldman, C. R. *Hydrobiologia* **251**, 39–47 (1993).
8. Addiscott, T. *New Scientist* **120**, 50–54 (1988).
9. Vitousek, P. M. *et al. Science* **204**, 469–474 (1979).
10. Carney, H. J. *Verh. int. Verein. Limnol.* **22**, 1253–1257 (1984).
11. Carney, H. J., Binford, M. W. & Kolata, A. L. in *Tiwanaku and its Hinterland*, Vol. 1: *Agroecology* (ed. Kolata A. L.) (Smithsonian Institution, in the press).
12. Zak, D. R. & Grigal, D. F. *Oecologia* **88**, 189–196 (1991).
13. Kolata, A. L. *Latin Amer. Antiquity* **2**, 99–125 (1991).
14. Binford, M. W., Brenner, M. & Engstrom, D. in *Lake Titicaca: A Synthesis of the Knowledge* (eds. DeJoux, C. & Iltis, A.) 29 (Kluwer, Dordrecht, 1992).
15. Brix, H. & Schierup, H.-H. *Ambio* **18**, 100–107 (1989).
16. *SAS User's Guide: Statistics P*, 119 (SAS Institute Inc., Cary, North Carolina, 1982).
17. Strickland, J. D. H. & Parsons, T. R. *A Practical Handbook of Seawater Analysis Bulletin* 167, 71 (Fisheries Research Board of Canada, Ottawa, 1972).
18. Strickland, J. D. H. & Parsons, T. R. *ibid* 53.
19. *Hach Water Analysis Handbook* 587 (Hach Company, Loveland, Colorado, 1989).

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Extraction of bound porphyrins from sulphur-rich sediments and their use for reconstruction of palaeoenvironments

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PORPHYRINS, which are present in most sediments and crude oils, represent the 'molecular fossils' of compounds such as chlorophylls, bacteriochlorophylls and haems in the organisms from which the organic material is derived^{1–4}. They have the potential to provide information about palaeoenvironmental conditions at the time of deposition^{5–9}. Porphyrins derived from degradation of chlorophylls are of particular interest because of the possibility of relating palaeoproductivity estimates from sediments to chlorophyll-based measurements of present-day productivity determined by remote sensing. But standard analytical methods do not detect all of the porphyrins present in a geological sample — a substantial fraction of the porphyrins may be bound to kerogen^{10,11} or to solvent-extractable macromolecules, or may be degraded by the oxidative extraction procedures. It has been shown recently^{12–16} that sulphur may play a crucial part in binding 'biomarker' molecules at an early stage of sediment diagenesis, and that desulphurization using Raney nickel may liberate small molecules bound to sulphur-containing species. Here we show that this approach releases large amounts of porphyrins from the total organic extract of a sulphur-rich marl. Liberating bound porphyrins in this way may greatly enhance the amount of information on palaeoenvironments that can be extracted from geochemical analysis of sediments.

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Porphyryns in sediments were first detected in 1934^{3,4}; later studies¹⁷ using mass spectrometry have demonstrated the complexity of petroporphyrin mixtures, and several groups have succeeded in isolating and characterizing pure compounds^{1-8,18,19}. The marl used in our studies was collected near Gibellina (Sicily) from an outcrop consisting of marly intercalations within gypsum conglomerates. These sediments are part of the Messinian formation (Upper Miocene, 5-7 Myr BP) which is characterized by thick evaporitic deposits over most of the Mediterranean basin²⁰. As no equivalents of these large scale evaporitic environments exist now, a geochemical study based on biological markers can be particularly useful in terms of understanding the sources and diagenesis of organic matter, as well as the conditions prevailing in these environments at the time of deposition^{21,22}. Furthermore, these series are potential source rocks of petroleum²⁰.

The sample investigated is highly immature as confirmed by Rock Eval pyrolysis data and contains 4% total organic carbon, more than a third (1.5% of total sediment) being extractable with organic solvent. The extract was composed mainly of sulphur-rich molecules and macromolecules. Chromatography of this total organic extract (Fig. 1) on silica gel gave only traces of free nickel and copper porphyrins (FNI+FCu) (<0.5 mg g⁻¹

of total organic extract; Fig. 2a) and no vanadyl porphyrins. Free bases were also detected (ultraviolet-visible spectroscopy) but, due to their chromatographic behaviour and instability, they could not be easily isolated. After treatment of the same extract with Raney nickel in refluxing toluene-ethanol (Fig. 1), a large amount (24 mg g⁻¹) of nickel porphyrins, including a minor fraction of copper porphyrins, were recovered (Fig. 2e; more polar porphyrins were also liberated and are currently under study). These nickel porphyrins may have arisen from Free-free bases (FfH₂), from Bound-free bases liberated by desulphurisation (BfH₂) (both being metallated by Raney nickel under the reaction conditions) or from Free (FNI) and Bound (BNI) nickel porphyrins. (Free and Bound indicate that porphyrins are free or bound to macromolecular organic matter; free bases is the chemical term for non-metallated porphyrins.)

In order to distinguish among these possible contributions, the total organic extract was treated with zinc acetate which complexes the free bases (Free or Bound) and separated in two equal aliquots (Fig. 1). In one case the Free nickel + copper (FNI+FCu) and the resulting zinc porphyrins (FfZn) were separated, the latter being then demetallated, and then remetallated with nickel (FfNi, Fig. 2c) in order to provide comparable HPLC profiles for all fractions. The second aliquot was treated with Raney nickel to liberate both Bound free bases (BfH₂), as zinc complexes (BfZn), and Bound nickel + copper porphyrins (BNI+BCu). A silica gel chromatographic separation gave the nickel + copper porphyrins (FNI+BNI+FCu+BCu, Fig. 2b) and the zinc porphyrins (FfZn+BfZn), the latter being in turn demetallated and remetallated with nickel (FfNi+BfNi, Fig. 2d). The relative

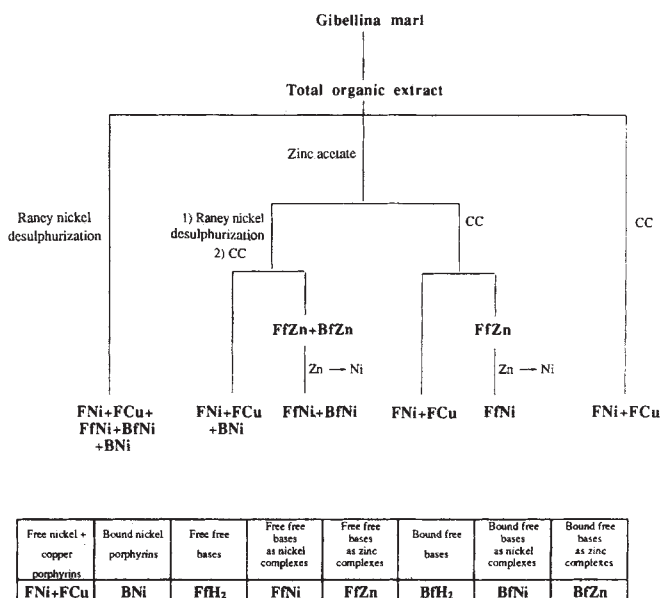


FIG. 1 Flow chart of the isolation procedure of the various porphyrin fractions from the Gibellina marl. The powdered marl was extracted (stirring for 2h in CHCl₃-methanol, 2h in toluene-methanol, and 2h in CHCl₃-methanol, all at 45 °C). Centrifugation and evaporation of the supernatant gave the total organic extract. The desulphurization experiments were run in refluxing toluene-ethanol 1:1. Raney nickel was washed with ethanol, then toluene, added to the solution and the suspension refluxed for 4h under hydrogen. The supernatant was decanted, the Raney nickel washed with CH₂Cl₂ and the combined organic phases evaporated, then dissolved in CH₂Cl₂ and filtered on celite. Metallation with zinc was performed by refluxing the extract in toluene-methanol in the presence of an excess of zinc acetate. Demetallation of zinc porphyrins occurred rapidly on addition of concentrated HCl to a CH₂Cl₂ solution, and nickel was inserted by refluxing the bases with nickel acetylacetonate in benzene (shown as Zn→Ni). Porphyrin fractions were isolated on silica gel column (CC) (eluent CH₂Cl₂-hexane 1:1), then purified on silica gel TLC (eluent CH₂Cl₂-hexane 1:3 for nickel porphyrins, 1:2 for zinc porphyrins). HPLC profiles and isolation of individual compounds were obtained on reverse phase RP-18 columns (eluent CH₂Cl₂-methanol 1:9)⁴.

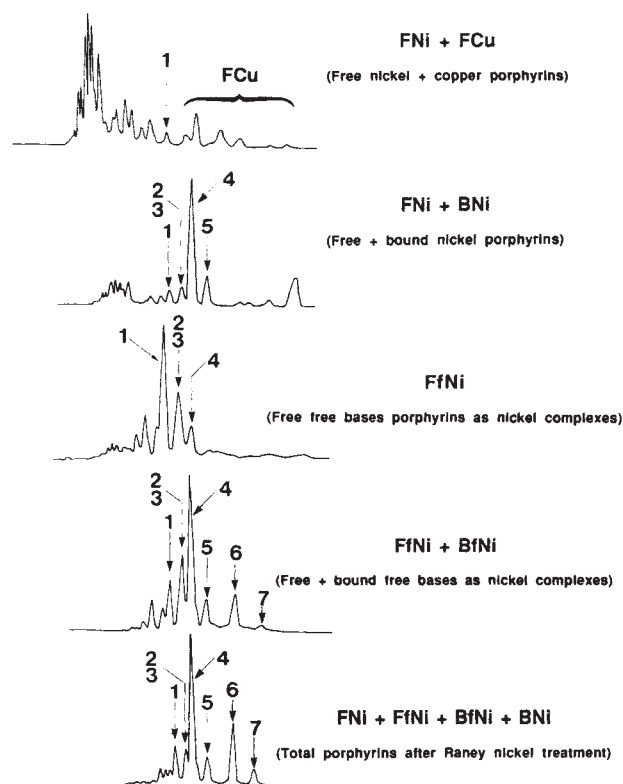


FIG. 2 HPLC profiles of various nickel porphyrin fractions obtained from Gibellina marl (copper porphyrins are only present in substantial amounts in the FNI + FCu fraction). HPLC conditions: RP-18 ODS Dupont, 4.6 × 250 mm, 1 ml min⁻¹, eluent CH₂Cl₂-methanol 1:9, detection 546 nm.

abundances of these various fractions could be obtained using ultraviolet-visible spectroscopy assuming that the relative absorptions are similar. We found that the free bases ($\text{FfH}_2 + \text{BfH}_2$) are more abundant than the complexed porphyrins ($\text{FNi} + \text{BNi} + \text{FCu} + \text{BCu}$) by a factor of 5, while the Bound porphyrins ($\text{BFH}_2 + \text{BNi} + \text{BCu}$) exceed the Free porphyrins ($\text{FNi} + \text{FCu} + \text{FfH}_2$) by a factor of 7. The contribution of copper porphyrins is the same order of magnitude as that of the nickel ones in the Free porphyrin fraction, while it is less than 5% in the Bound porphyrin fraction. These results demonstrate that, for the Gibellina marl, a classical approach (extraction followed by simple chromatographic separation of the porphyrins) would miss most of the useful compounds and the related geochemical information. The alkanes from the same sediment show the same behaviour (20:1 ratio between bound and free hydrocarbons) and a totally different distribution which is in agreement with previous observations on other samples^{15,16}. We do not know how sulphur participates in the bonding of porphyrins to larger entities, but model reactions indicate that several positions may be involved²³.

Because Raney nickel is a powerful reducing agent we feared that some porphyrins might have been reduced and subsequently degraded and lost. To test this possibility on a variety of petroporphyrin structures simultaneously, a series of experiments was run under the conditions used for Gibellina marl using a geochemical porphyrin mixture (as nickel, vanadyl, but also zinc complexes or free bases, prepared from the nickel complexes) from Julia Creek oil shale (Lower Cretaceous, Australia)²⁴. When treated with Raney nickel each porphyrin mixture underwent immediate and irreversible discolouration, indicating that the reaction did not stop at the reduction at the meso-bridge positions. In contrast, the same reaction run in the presence of the Gibellina total organic extract led to the complete recovery of the Julia Creek starting material, the free bases being quantitatively metallated with Raney nickel as expected. The vanadium, nickel, as well as the sensitive zinc complexes were recovered intact, as well as the nickel porphyrins from the Gibellina extract (there is very little interference, in terms of porphyrin HPLC profiles, between the Gibellina and the Julia Creek samples). It appears that some constituents of the total organic extract exert a "protective effect" on the porphyrin nuclei. We speculate that the abundance of sulphur and sulphur-containing compounds, as well as the presence of a wide variety of structures in the total organic extract, might neutralize the most active sites of the Raney nickel and lead to the high selectivity observed. These results suggest that previous studies run on prefractionated (chromatographic separation^{12-14,16} or selective precipitation¹⁵) geochemical mixtures may suffer from severe biases. This was clearly illustrated when we first chromatographed the total extract: none of the fractions containing porphyrins, as shown by their visible absorption, yielded detectable amounts of porphyrins after Raney nickel treatment, as observed with the Julia Creek porphyrin fractions.

The structural investigation of the major components from the marl (Fig. 3) confirmed that the search for bound porphyrins is essential in this sulphur-rich sample: the distribution in fraction $\text{FNi} + \text{FCu}$ (the only one that would have been easily obtained using classical procedures, but also the smallest one) is totally different from the three other fractions. Indeed, the $\text{FNi} + \text{FCu}$ fraction shows a dominance of the simple alkylporphyrins over the cycloalkanoporphyrins (FNi ; alkylporphyrins from C_{27} to C_{32} dominate) or the opposite (FCu ; deoxophylloerythroetioporphyrins (DPEPs) from C_{30} to C_{32} dominate), as measured by mass spectrometry. On the other hand, the Free-free bases (FfH_2) as well as the Bound porphyrins (free bases, nickel, and copper complexes) are dominated by cycloalkanoporphyrins. The major compounds detected in the Free-free bases fractions are classical DPEP's 1-3 (in Fig. 3) and traces of 4. More striking is the fact that in both fractions

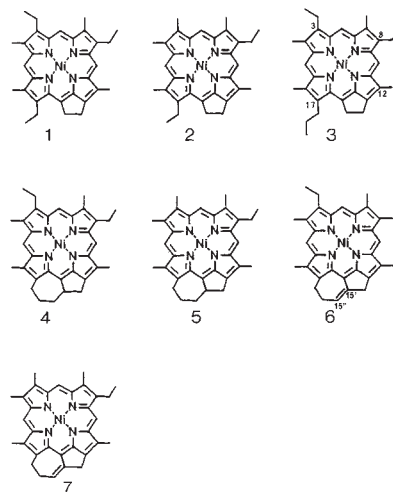


FIG. 3 Typical porphyrins found in Gibellina marl (see Fig. 2). These compounds were purified on reverse phase HPLC, and characterized by their mass spectra and NMR data (400 MHz in deuterated benzene) and comparison with reference samples.

liberated by desulphurization, the major porphyrins are compounds 4-7. Porphyrins with the 5+7 membered ring system have been detected in several sediments²⁵⁻²⁸ but as minor components. The evaluation of their contribution may however be underestimated due to their relative instability (P. Sundaraman, personal communication). This petroporphyrin series clearly originates from chlorophylls and could be a product of grazing of phytoplankton by marine organisms. Some evidence for this is provided by the recent isolation, from a sponge and a clam, of chlorophyll metabolites possessing the same carbon framework²⁹⁻³¹. The 3-methyl counterparts of 1 and 4 (2 and 5 respectively), and 17-propyl DPEP (3) are present, 2 and 3 being usually found in carbonate-containing sediments deposited under anoxic conditions⁴. Compounds 6 and 7 (15',15"-dehydro) are only present in the Bound-free bases fraction. They were reduced to their saturated counterparts 4 and 5 when the desulphurisation was run under a hydrogen atmosphere (the same phenomenon was observed in the case of the hydrocarbon fraction). It is tempting to suggest that a sulphur atom was therefore attached to position 15' or 15".

The Gibellina marl was deposited under evaporitic conditions, and was probably quite specialized in terms of biological activity^{21,22}. Most likely the deposition took place under an anoxic water layer due to the development in the bottom waters of sulphate reducing bacteria. Evidence that the water column was stratified and that the chemocline probably reached into the photic zone could be provided from the occurrence in the bound hydrocarbon fraction of molecular fossils of aromatic carotenoids typical of green sulphur bacteria³² (the corresponding porphyrins homologated at positions 8 and 12 could, however, not be detected in significant amounts). An important algal contribution coming from aerated surface waters obviously took place, as shown for example by the strong predominance of steroids in the bound hydrocarbon fractions.

Porphyrins 4-7, which are present in the highest concentrations, could either be metabolites of chlorophylls from phy-

toplankton blooming in the aerated surface water layers or degradation products of bacteriochlorophylls from photosynthetic bacteria growing below the oxic/anoxic interface (for example bacteriochlorophylls *a* and *b*). Whatever their origin, these relatively unstable intermediates survived diagenetic conditions and were preserved in the protective anoxic zone. This preservation is most likely due to their binding into macromolecular entities by reaction with inorganic sulphur species which occurred in the anoxic bottom part of the basin as a result of anaerobic microbial activity. The high concentration of free bases could result from the effective absence of complexing metals in the sulphur rich anoxic zone because most of the usual metals (Ni, Cu, Fe) would be quenched as sulphides. It is therefore tempting to suggest that the Free (Ni + Cu) porphyrins are mainly derived from phytoplankton from the aerated layers where metal ions were still present; as large sediment samples represent 'averages' of depositional conditions, an alternative explanation could be that these porphyrins have been deposited during times when the water column was aerated down to the sediment. The high concentrations of porphyrins 4–7 with a 7+5 ring system and their occurrence as free bases could therefore represent typical features characteristic of this type of evaporitic environment. □

Calculating erosion by deep-sea turbidity currents during initiation and flow

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TURBIDITY currents transport massive amounts of sediment from continental margins to the deep sea. Individual flows can catastrophically remove and redeposit (as turbidites) many hundreds of cubic kilometres of material^{1,2}, with the larger events reaching the bottom of the continental slope to form the abyssal plains³. Here we show that the age range of sediments in individual turbidites can be used directly to estimate both the thickness of failed sediment in the source region (even when its exact location is unknown) and the extent to which the turbidity current caused erosion of the sea bed. Our method involves the comparison of the abundance ratios of microfossil (coccolith) species in turbidites with those in the ocean margin sediments of the source region. Analysis of a recently emplaced turbidite on the Madeira Abyssal Plain shows that it contains a mixture of sediments with an age range of about 200,000 years, equivalent to the failure of a block of sediment about 15 m deep. Radiocarbon dating and coccolith ratios show that the turbidite contains only about 12% of recent, near-surface sediment, indicating that this turbidity current caused surprisingly little erosion en route.

Few turbidity currents have been observed directly, so studies of these events rely largely on laboratory simulations and mathematical models^{4,5}. The essential criterion for a turbidity current is that the sediment is held in suspension by the upward component of fluid turbulence⁶. If the flow velocity wanes, sediment will be deposited and the flow will decelerate further. If the velocity is high enough to erode, however, the volume of the turbidity current will increase, leading to a gain in velocity and a potential increase in erosive power⁷.

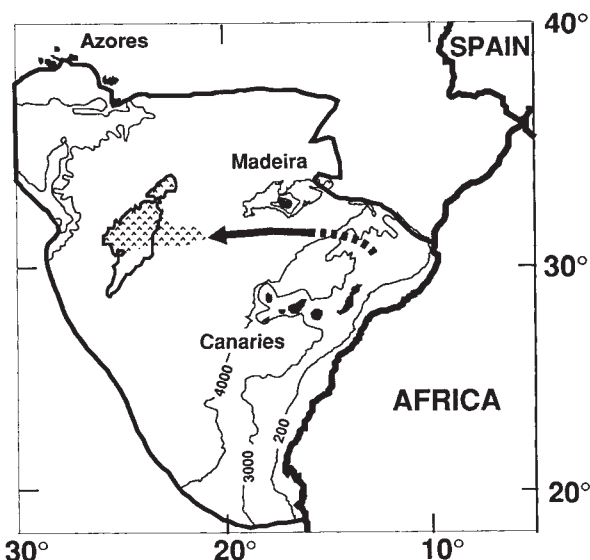


FIG. 1 Map of the Canary basin showing the Madeira Abyssal Plain and the area now covered by turbidite *a* (patterned region). Source direction of the turbidite is indicated by the arrow, although the exact source has not been located.

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- Ocampo, R., Callot, H. J. & Albrecht, P. in *Metal Complexes in Fossil Fuels: ACS Symposium Series 344* (eds Filby, R. H. & Branthaver, F. J.) 68–73 (American Chemical Society, Washington DC, 1987).
- Chicarelli, M. I., Kaur, S. & Maxwell, J. R. *ibid.* 41–67.
- Callot, H. J., Ocampo, R. & Albrecht, P. *Energy & Fuels* **4**, 635–639 (1990).
- Ocampo, R., Bauder, C., Callot, H. J. & Albrecht, P. *Geochim. cosmochim. Acta* **56**, 745–761 (1992).
- Ocampo, R., Callot, H. J., Albrecht, P. & Kintzinger, J. P. *Tetrahedron Lett.* **25**, 2589–2592 (1984).
- Ocampo, R., Callot, H. J. & Albrecht, P. *JCS Chem. Commun.* 200–201 (1985).
- Verne-Mismer, J., Ocampo, R., Callot, H. J. & Albrecht, P. *Tetrahedron Lett.* **29**, 371–374 (1988).
- Bauder, C., Ocampo, R., Callot, H. J. & Albrecht, P. *Naturwissenschaften* **77**, 378–379 (1990).
- Hayes, J. M., Takigiku, R., Ocampo, R., Callot, H. J. & Albrecht, P. *Nature* **329**, 48–51 (1987).
- Blumer, M. & Snyder, W. D. *Chem. Geol.* **2**, 35–45 (1967).
- Sundaraman, P., Biggs, W. R., Reynolds, J. G. & Fetzer, J. C. *Geochim. cosmochim. Acta* **52**, 2337–2341 (1988).
- Schmid, J. C. thesis, Univ. Louis Pasteur, Strasbourg (1986).
- Schmid, J. C., Connan, J. & Albrecht, P. *Nature* **329**, 54–56 (1987).
- Sinninghe-Damste, J. S. & De Leeuw, J. W. *Org. Geochem.* **16**, 1077–1101 (1990).
- Kohnen, M. E. L., Sinninghe-Damste, J. S., & De Leeuw, J. W. *Nature*, **349**, 775–778 (1991).
- Adam, P. *et al. Geochim. cosmochim. Acta* (in the press).
- Baker, E. W. *J. Am. chem. Soc.* **88**, 2311–2315 (1966).
- Quirke, J. M. E., Eglinton, G. & Maxwell, J. R. *J. Am. chem. Soc.* **101**, 7693–7697 (1979).
- Quirke, J. M. E., Maxwell, J. R., Eglinton, G. & Sanders, J. K. M. *Tetrahedron Lett.* **21**, 2987–2990 (1980).
- Palmer, S. E. & Zumberge, J. E. in *Organic Maturation Studies and Fossil Fuel Exploration* ed. Brooks, J. 392–426 (Academic London, 1981).
- Javor, B. *Hypersaline Environments* (Springer, Berlin, 1989).
- Eugster, H. P. *Geochim. cosmochim. Acta* **49**, 619–635 (1985).
- Rohrer, A., Ocampo, R. & Callot, H. J. in *204th American Chemical Society National Meeting Division of Geochemistry*, Abstract 3 (American Chemical Society, Washington, 1992).
- Boreham, C. J. & Fookes, C. J. R. *J. Chromatogr.* **467**, 195–208 (1989).
- Chicarelli, M. I. & Maxwell, J. R. *Tetrahedron Lett.* **38**, 4653–4654 (1986).
- Prowse, W. G., Chicarelli, M. I., Keely, B. J., Kaur, S. & Maxwell, J. R. *Geochim. cosmochim. Acta* **51**, 2875–2877 (1987).
- Keely, B. J. & Maxwell, J. R. in *14th Int. Meeting on Organic Geochemistry*, Abstract 179. (European Association of Organic Geochemists, Paris, 1989).
- Verne-Mismer, J., Ocampo, R., Bauder, C., Callot, H. J. & Albrecht, P. *Energy & Fuels* **4**, 639–643 (1990).
- Karuso, P. *et al. Tetrahedron Lett.* **27**, 2177–2178 (1986).
- Sakata, K. *et al. Tetrahedron Lett.* **31**, 1165–1168 (1990).
- Yamamoto, K. *et al. Tetrahedron Lett.* **33**, 2587–2588 (1992).
- Repeta, D. J., Simpson, D. J., Jorgensen, B. B. & Jannasch, H. W. *Nature* **342**, 69–72 (1989) and references therein.

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TABLE 1 Calculations of the predicted radiocarbon age

Oxygen isotope stage*	Time period (kyr)	Duration (kyr)	Effective mid-point of stage (kyr)†	Effective $e^{-\lambda t}$ for stage‡
1	1 ⁶ -12	11	5.88	
2	12-24	12	17.27	0.4810
3	24-59	35	35.92	0.1165
4	59-71	12	64.27	0.0114
5	71-128	57		0.0003
6	128-186	58		

Ages are calculated on the assumption that turbidite *a* incorporates sediment accumulated over ~200,000 years. The predicted radiocarbon age of a composite of sediment from isotope stages 1–6, weighted according to stage duration and assuming a constant accumulation rate through time, is $\ln((\sum \text{mean } e^{-\lambda t} \text{ of stage} \times \text{stage duration})/185)/\lambda = 26,200 \text{ yr}$. If V_1 is the initial volume of the turbidite at source, V_2 is the additional volume of surficial material incorporated during transport, and t_1 , t_2 and t_3 are the mean ages of the turbidite material at source (26,000 yr), incorporated surficial material (4,500 yr)§ and the final turbidite material (18,345 yr), respectively, then $(V_1 \times e^{-\lambda t_1}) + (V_2 \times e^{-\lambda t_2}) = (V_1 + V_2) \times e^{-\lambda t_3}$, whence $V_1 = 7.38 \times V_2$.

*Oxygen isotope stage boundaries according to Imbrie *et al.*²².

†Effective mid-point of stage calculated according to Hoffman and van Amerik²¹.

‡ $\lambda = \ln 2/5568$ (Libby radiocarbon half-life).

§Turbidite emplaced 1,000 years ago¹¹, so the past 1,000 years is absent from the turbidite, and surficial sediment incorporated during the flow now has an age of $3,500 + 1,000 \text{ yr} = 4,500 \text{ yr}$.

The Madeira Abyssal Plain^{8–10} comprises a sequence of large-volume turbidites derived from the northwest African margin and Canary Islands. The most recently emplaced unit (turbidite *a*) was deposited 1,000 years ago¹¹ and has an estimated volume of 25 km³. It is primarily a fine-grained unit (median grain size 1.5 μm), composed of nannofossils and clay particles, with a basal foraminiferal sand at the proximal edge of the plain. Its distribution and composition suggest a source north of the Canary Islands, and a transport path at least 700 km long across the northwest African continental rise¹⁰ (Fig. 1).

The likely source areas for turbidite *a* (Fig. 1) are characterized by hemipelagic sequences with no turbidites¹². Coccoliths (calcareous skeletons of microscopic algae) are extremely abundant in these sediments, but the abundance of different species varies with time, with single species dominating the flora for short time intervals (acmes). These acmes follow a consistent pattern over most of the temperate North Atlantic¹³. Successive changes in the ratios of the five dominant species throughout the past 550 kyr are shown in Fig. 2. When a turbidity current is initiated, sediments containing part of this succession are eroded and species mixtures typical of several acme zones are combined to produce a new mixture of coccolith species, different from any found in pelagic or hemipelagic sediments. In principle it should be possible to simulate the exact mixture by comparing the species ratios observed in the turbidite with synthetic ratios created by mixing increasingly older layers from the hemipelagic record. This requires a knowledge of the geometry of the failed sediment volume and its accumulation history. Mapped slope failures such as the Saharan slide¹⁴ and the Storregga slide¹⁵ suggest that sediments usually fail as slabs with steep sides and flat floors. Accumulation rates off northwest Africa have been relatively constant through the past 10⁶ years^{12,16}, and the assumption is made that the failed volume is a rectangular unit accumulated at constant rate. Such slope failures cannot omit layers unless hiatuses were present in the eroded block.

A comparison of coccolith ratios observed in turbidite *a* with synthetic mixtures of ratios from successively older sediment layers is shown in Fig. 3. The ratio observed in turbidite *a* compares well with the synthetic mixture of isotope stages 1–6. This represents hemipelagic accumulation between now and 186,000 years ago, which is equivalent to the age range of sediment in the turbidite. The data are consistent with a slab-shaped eroded mass, and we have found they do not fit other models, for example a wedge-shaped eroded mass.

Knowing the age range of sediment within the turbidite, we can now calculate more of its characteristics. For a mean hemipelagic accumulation rate in the source area of 8 cm kyr⁻¹ (ref. 12), the depth of erosion required to form the turbidite would be 15 m. The volume of turbidite on the plain is about 25 km³, so an area of erosion of about 1,700 km² would be required in the source area. Once generated, the turbidity current flowed at least 700 km to the abyssal plain over sediments of Holocene age with ratios of coccolith species very different to those of the turbidite mixture (Figs. 2 and 3). If the turbidity current had significantly eroded these upper layers its coccolith mixture would shift to contain greater numbers of *Emiliania huxleyi*. Figure 3 shows that this has not been the case, and we conclude that the turbidity current was virtually non-erosive over most of its pathway.

To test this surprising conclusion, we determined the radiocarbon age of the calcium carbonate (mainly coccoliths) in the body of the turbidite. The weighted mean radiocarbon age from six analyses is $18,345 \pm 63$ years (G.T. Cook, personal communication). For comparison, the predicted radiocarbon age of a composite of sediment from isotope stages 1–6, weighted according to stage duration with uniform accumulation rate, is 26,200 years (Table 1). Some younger material must therefore have been incorporated into the turbidite to account for the age difference. If it is assumed that this input occurred during transport, surficial sediments along the flow path would be the most exposed and easily eroded material, as well as having the youngest ¹⁴C age. The combination of the low accumulation rate in this mid-gyre region (2–3 cm kyr⁻¹) and bioturbation at the sediment–water interface^{17–19} results in a

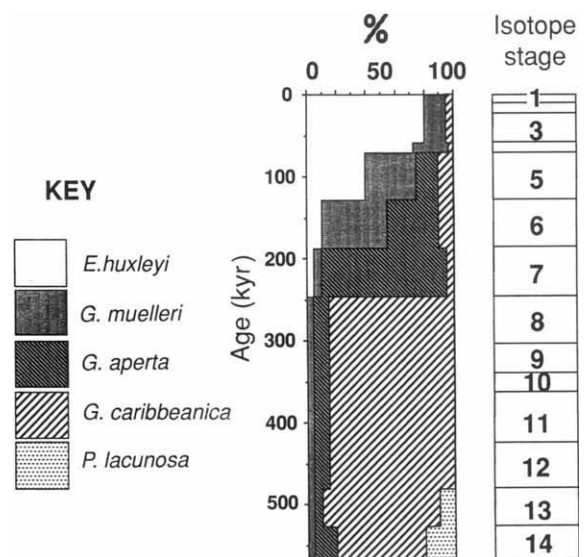


FIG. 2 Ratios of the most abundant coccoliths found in sediments from the past 550,000 years¹³. Note the turnover of dominant species (acmes) and the extinction of *Pseudoemiliana lacunosa* (this extinction actually occurs in oxygen isotope stage 12 but its percent abundance is almost negligible in this stage). Percentages are given as averages for each isotope stage.

surface mixed-layer age of ~ 3.5 kyr in the uppermost ~ 5 cm of the sediments²⁰. A contribution of 12% of such surficial material to the total turbidite volume is required to account for the discrepancy between the predicted and measured radiocarbon ages (Table 1). Although it would be possible to obtain the younger age by adding sediments from below the surface mixed layer, any greater depth of erosion would require much larger volumes of sediment because of the short half-life of ^{14}C (5,568 years). The 12% addition adds only 4% to the *E. huxleyi* values, but any greater volume would increase the ratio of *E. huxleyi* significantly beyond the measured values (Fig. 3).

The total volume of turbidite *a* is estimated at 25 km^3 , of which 3 km^3 could have been added during transport. It is not possible to determine exactly where the excess surface material would be picked up; this could be around the source area, over the whole transport pathway or irregularly along the pathway. We do not know the width of the flow, but estimate a maximum of $\sim 100 \text{ km}$ based on local topography, making the area crossed by the flow less than $70,000 \text{ km}^2$ (Fig. 3). If the sediment was picked up equally over this whole area the average depth of erosion would be about 4.3 cms. It is more likely that this surficial material was eroded soon after the turbidity current was formed, near the source area where accumulation rates are higher and the volume required could be achieved by deeper erosion of a smaller area. The original estimate of an eroded area of $17,000 \text{ km}^2$ must be decreased by 12% to account for the material eroded during flow, and now becomes $\sim 15,000 \text{ km}^2$.

Our micropalaeontological method of age analysis of turbidite mixtures gives estimates of depth and shape of eroded bodies consistent with field data. Comparison of the results with independent ^{14}C dating confirms that the turbidite is composed mainly of sediment from the source area with up to 12% of younger sediment added through erosion of the sea bed during flow of the turbidity current. The micropalaeontological method of assessing the age range of sediment in a turbidite will be applicable to any turbidite where the hemipelagic coccolith

record of the source material is known in detail, especially where species abundances change with time. To convert age range to depth of erosion the hemipelagic accumulation rate in the source area must be known, and to determine the area of erosion it is necessary to know the volume of the turbidite. These conditions are most likely to be met in modern deep-sea basins or in ancient basins that have been thoroughly studied. Testing against ^{14}C data will only be possible for turbidites deposited within the past few tens of thousands of years, but should provide valuable data for determining erosion in basins with different geometries, such as steep slopes. □

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- Piper, D. J. W., Shor, A. N., Farre, J. A., O'Connell, S. & Jacobi, R. *Geology* **13**, 538–541 (1985).
- Heezen, B. C. & Ewing, M. *Am. Assoc. Petrol. Geol. Bull.* **39**, 2505–2514 (1955).
- Weaver, P. P. E. & Thomson, J. (eds) *Geology and Geochemistry of Abyssal Plains* (Geol. Soc. Lond. spec. Publ. 31, Blackwell, Oxford, 1987).
- Simpson, J. E. A. *Rev. Fluid Mech.* **14**, 213–234 (1982).
- Pantin, H. M. *Mar. Geol.* **31**, 59–99 (1979).
- Bagnold, R. A. *Proc. R. Soc. A* **265**, 315–319 (1962).
- Parker, G., Fukushima, Y. & Pantin, H. M. *J. Fluid Mech.* **171**, 145–181 (1986).
- Weaver, P. P. E. & Kuijpers, A. *Nature* **306**, 360–363 (1983).
- Weaver, P. P. E. & Rothwell, R. G. in *Geology and Geochemistry of Abyssal Plains* (eds Weaver, P. P. E. & Thomson, J.), 71–86 (Geol. Soc. Lond. spec. Publ. 31, Blackwell, Oxford, 1987).
- Weaver, P. P. E., Rothwell, R. G., Ebbing, J., Gunn, D. & Hunter, P. M. *Mar. Geol.* **109**, 1–20 (1992).
- Thomson, J. & Weaver, P. P. E. *Sedim. Geol.* (in the press).
- Koopmann, B. *"Meteor" Forsch-ergerbn.* **C35**, 23–59 (1981).
- Weaver, P. P. E. & Hine, N. in *Stratigraphic Index of Calcareous Nannofossils* (eds A. R. Lord & P. Bown) (British Micropalaeont. Soc. spec. Publ., in the press).
- Embley, R. W. in *Marine Slides and Other Mass Movements* (eds Saxov, S. & Nieuwenhuis, J. K.) 189–213 (Plenum, New York, 1982).
- Bugge, T. *Cont. Shelf Inst., Norway, Publ.* **110**, (1983).
- Ruddiman, W. et al. *Proc. ODP Sci. Res.* **108** (Ocean Drilling Program, College Station, Texas, 1989).
- Thomson, J., Colley, S. & Weaver, P. P. E. *Earth planet. Sci. Lett.* **90**, 157–173 (1988).
- Erlenkeuser, H. *Earth planet. Sci. Lett.* **47**, 319–326 (1980).
- Officer, C. B. *Mar. Geol.* **42**, 261–278 (1982).
- Kershaw, P. J. *J. env. Radioactivity* **2**, 145–160 (1985).
- Hoffman, B. W. & van Camerik, S. B. *Analyt. Chem.* **39**, 1198–1199 (1967).
- Imbrie, J. et al. in *Milankovitch and Climate* (eds Berger, A., Imbrie, J., Hays, J., Kukla, G. & Saltzman, B.) 269–305 (NATO ASI series 1984).

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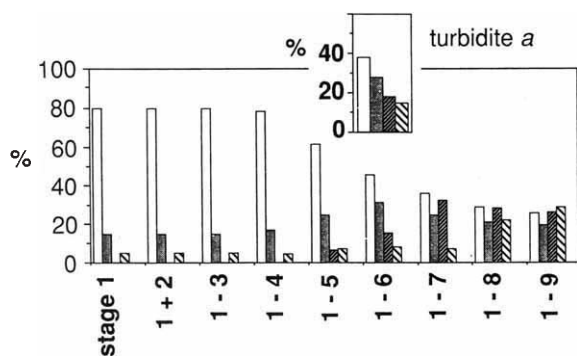


FIG. 3 Synthetic ratios of coccolith species for mixtures incorporating material from oxygen isotope stage 1 only, through to a mixture incorporating all isotope stages from 1–9. For key to shading, see Fig. 2. For each case we have assumed a constant accumulation rate in the source area and equal volumes of material contributed for each time interval (kyr). The synthetic mixtures were created by taking the average values of the species percentages for each isotope stage from Fig. 1 and multiplying these by the length of the stage. For each mixture the appropriate number of isotope stages were added and a new percentage calculated. Inset: Measured ratio of species in turbidite *a* from the Madeira Abyssal Plain. These ratios are very similar to the synthetic mixture of isotope stages 1–6. *P. lacunosa*, common in sediments older than oxygen isotope stage 12 (450 kyr ago) is not present in turbidite *a*, indicating that the turbidite contains only younger material. If 12% of the sediment was added during transport from the surficial layer (as predicted in the text) the ratio of *E. huxleyi* in the mixture of isotope stages 1–6 would increase from 45.5% (main panel) to 49.5%, compared to the measured values of 38% (inset panel).

The displacement field of the Landers earthquake mapped by radar interferometry

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GEODETIC data, obtained by ground- or space-based techniques, can be used to infer the distribution of slip on a fault that has ruptured in an earthquake. Although most geodetic techniques require a surveyed network to be in place before the earthquake^{1–3}, satellite images, when collected at regular intervals, can capture co-seismic displacements without advance knowledge of the earthquake's location. Synthetic aperture radar (SAR) interferometry, first introduced⁴ in 1974 for topographic mapping^{5–8} can also be used to detect changes in the ground surface, by removing the signal from the topography^{9,10}. Here we use SAR interferometry to capture the movements produced by the 1992 earthquake in Landers, California¹¹. We construct an interferogram by combining topographic information with SAR

images obtained by the ERS-1 satellite before and after the earthquake. The observed changes in range from the ground surface to the satellite agree well with the slip measured in the field, with the displacements measured by surveying, and with the results of an elastic dislocation model. As a geodetic tool, the SAR interferogram provides a denser spatial sampling (100 m per pixel) than surveying methods¹⁻³ and a better precision (~3 cm) than previous space imaging techniques^{12,13}.

The magnitude 7.3 (M_w) Landers earthquake of 28 June 1992 ruptured over 85 km along a fault system that included the Johnson Valley, Homestead Valley, Emerson, and Camp Rock faults (Fig. 1). Field¹¹ and seismological¹⁴ investigations show right-lateral slip reaching maxima of 4 m and 6 m, respectively 10 km and 40 km north of the main shock, for which the hypocentral depth¹¹ was between 3 and 8 km. This event was followed 3 hours later by the Big Bear earthquake (M_w 6.2), for which no surface rupture was reported. Co-seismic horizontal displacements as large as 3 m were measured geodetically and are in good agreement with simple elastic dislocation models^{1-3,15}. Near the fault, co-seismic displacements of the order of a metre were detected by pixel correlation of SPOT satellite images¹³ and up to 6 cm of post-seismic displacement was

observed by surveys in the month following the earthquake¹⁶. The sequence of earthquakes altered the state of stress on the San Andreas fault system^{17,18} and triggered seismicity elsewhere in North America¹⁹.

The ERS-1 satellite passes over the rupture area (Fig. 1) at an altitude of 785 km, transmitting along ray paths pointed west at an average angle of 23° from the vertical. Each SAR image is a map of the ground reflectivity sorted by range, the distance from the radar antenna to the ground. The phase of each 4 by 20 m pixel measures both the range and the phase shift due to reflection of the wave from the ground surface. The latter quantity can be eliminated between two images of the same area if the dielectric characteristics of the ground remain constant and the orbits satisfy the conditions necessary for coherence^{6,20}. The remaining path difference, known only to within an integer number of wavelengths, contains information from three sources: (1) relative orbital positions, (2) topography as seen in stereo by the satellite from slightly different orbital passes and (3) any change in position of the ground reflector between the acquisition times of the two images^{8,10}.

SAR images of the rupture zone were acquired by ERS-1 on four separate dates in 1992: 24 April, 3 July, 7 August, and 11 September. Among the three pairs spanning the earthquake date, the April–August 7 pair provides the optimum conditions for image correlation because the orbital separation best meets the coherence condition and the reflective surface was well preserved despite the intervening 105 days.

From these images, we reconstruct the phase of each pixel using a phase-preserving correlator²¹. We adjust the satellite orbital parameters (1) to minimize the number of fringes at the four corners of the image, assuming that the far field displacement is negligible. The stereoscopic path difference (2) is eliminated using a digital elevation model²². The interferometric fringes (3) are calculated in the geometry of the radar image and then mapped into the cartographic geometry. There they are resampled on the 90 by 110 m pixels of the elevation model to improve the signal-to-noise ratio.

The resulting interferogram (Fig. 2a and b) is a contour map of the change in range, that is the component of the displacement which points toward the satellite. It includes all the co-seismic and some of the post-seismic deformation. Each fringe corresponds to one cycle, equivalent to 28 mm (half the 56-mm wavelength of the ERS-1 SAR). For the nine geodetic stations located in the coherent part of the interferogram, the range changes are comparable to those calculated from surveying observations of horizontal displacement¹⁻³ with an r.m.s difference of 1.2 cycles, or 34 mm. This value represents the uncertainty, in an absolute sense, of the range change for a given point in the interferogram, and probably reflects mostly orbital errors.

Figures 2b and c shows two different interferograms of the same area near the fault. Figure 2c was processed in the same way as Fig. 2b, but using a pair of images taken after the earthquake on 3 July and 7 August. Comparison of these two images clearly shows that the fringes in Fig. 2a and b are due to the earthquake. The comparison also indicates that errors in the elevation model propagate into the ranges in Fig. 2a and b at the level of 9 mm, as quantified in the caption. This value represents the uncertainty of the relative change in range between two nearby points in the interferogram.

The co-seismic interferogram (Figs. 2a, 2b and 3a), shows no organized fringes in a band within 5–10 km of the fault trace. In this area, the displacement gradient is sufficiently large that the change in range across a radar pixel exceeds a critical value, and coherence is lost^{6,20}. Rotations of small crustal blocks may also reduce the coherence²³. Indeed, the band of incoherence is not observed in the post-earthquake interferogram (Fig. 2c).

For comparison, we calculate the theoretical change in range (Fig. 3b) using a dislocation model which describes the rupture zone as an elastic half space¹⁵. The earthquake fault is treated as

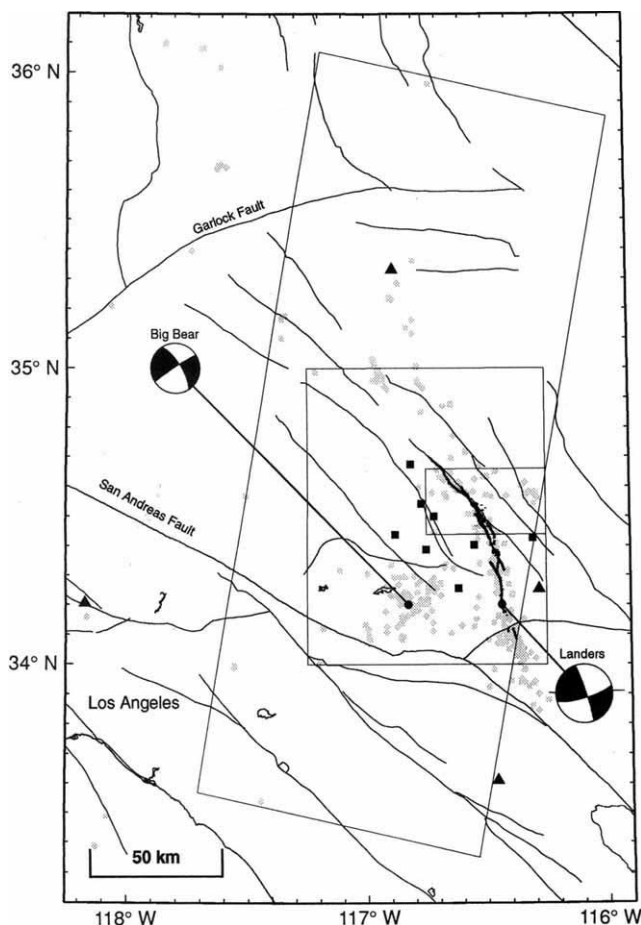


FIG. 1 Active faults in the region of the Landers and Big Bear earthquakes. Heavy solid lines indicate surface rupture associated with the Landers event¹¹. No surface break was reported for the Big Bear event. Solid circles are epicentres¹¹ of Landers and Big Bear main shocks with focal mechanisms (G. Ekström and M. Salganik, personal communication). Gray spots are earthquakes with magnitude greater than 3 between 25 June and 8 August 1992. Rectangles denote the areas covered by Fig. 2a (large), Fig. 2b and c (small) and Fig. 3a and b (medium). Squares and triangles denote geodetic stations where the co-seismic displacement has been estimated by the U.S. Geological Survey³ and the Permanent GPS Geodetic Array^{1,2}, respectively.

eight vertical planar segments which represent the surface rupture and the aftershock distribution¹¹. Each segment is subdivided into rectangular patches 2 km in length. For the Landers event, the slip on each patch matches the offset mapped in the field (K. Hudnut, personal communication, published in ref. 14). The modelled rupture extends from 0 to 15 km below the surface, to match the moment¹¹ of 10^{20} N m. For the Big Bear event, where no surface rupture was observed, the model includes the geometry from previous studies^{1,2}, a simple triangular slip distribution extending from 3 to 15 km below the surface, and a moment² of 4×10^{18} N m.

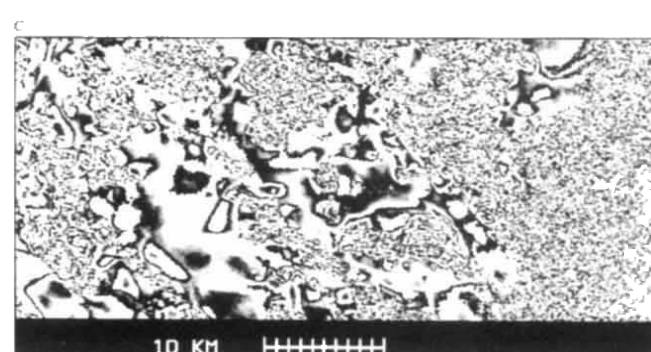
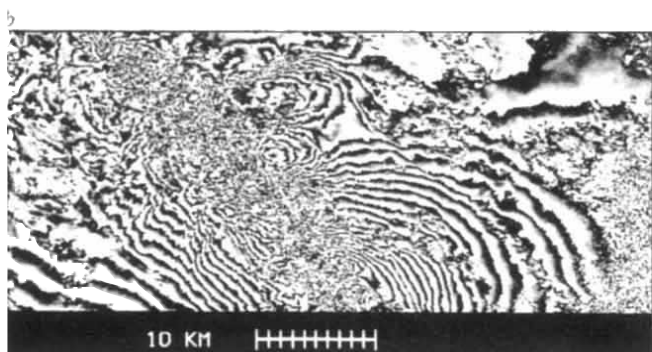
The outstanding resemblance between the modelled and observed fringe patterns, which were obtained independently, validates both calculations. They agree to within two fringes (56

mm) in both the near and far field. Larger, but local, differences within 10 km of the fault are due to the simple discretization of the elastic model. The large lobes are adjacent to the central section of the fault, where the maximum slip was observed¹¹. On both sides of the fault, the fringes converge toward points where the fault slip vanishes. This occurs at both ends of the fault and 20 km north of the epicentre, between the two main ruptures^{11,14}.

The modelled and observed changes in range agree to within 30 mm in a profile perpendicular to the central section of the fault (Fig. 4a). The agreement in the decay of displacement with distance out to 40 km from the fault suggests that the rupture depth of 15 km in the model is correct in this section of the fault. The right-lateral slip on the fault can be estimated from the



FIG. 2 *a*, Post-processing interferometric fringes obtained with the pair of ERS-1 SAR images taken before (24 April) and after (7 August) the earthquake. The image covers the 125 by 275 km area outlined by the large rectangle in Fig. 1. One cycle of gray shading represents a range difference of 28 mm between the two images. It is a measure of the component of the displacement vector which points toward the satellite. The number of fringes increases from zero at the northern edge of the image, where no co-seismic displacement is assumed, to at least 20 (representing 560 mm in range difference) in the cores of the lobes adjacent to the fault. The asymmetry between the two sides of the fault is due to the curvature of the fault and the geometry of the radar. Displacements with different azimuths on opposite sides of the fault produce different fringe patterns because the radar resolves only the range component of the displacement vector. *b*, Detail of the fringe map in *a*, covering an area 64 km by 33 km as outlined by the small rectangle in Fig. 1. The orbital separation, or baseline, between the images taken 24 April and 7 August measures approximately 60 m and 126 m, in the vertical and horizontal components respectively. These values imply that an error in the elevation model of 72 m would produce a shift of one full cycle. *c*, Fringes obtained using the same processing and area for *b*, but with SAR images acquired on 3 July and 7 August (after the 28 June earthquakes). The maximum post-seismic displacement observed¹⁶ in the area and time covered by this image pair is 4 cm, which would produce a change in range of less than 0.2 cycles. The fringes are apparently due to errors in the elevation model because the interferogram exhibits characteristic topographic fringes and local incoherence in areas of high relief²⁰. The orbital separation of this pair of images measures 22 m and 496 m in the vertical and horizontal components, respectively. These values imply that an error of 16 m in the elevation model would create one fringe. This interferogram is thus 4.5 times more sensitive to such errors than the co-seismic image pair (*a* and *b*). Since this post-seismic image pair (*c*) exhibits a noise level of about 1.5 cycles, we infer about 24 m of noise in the elevation model, in agreement with the published uncertainty²² of 30 m. This noise level would in turn contribute about one third of a cycle, or 9 mm to the range changes in the co-seismic interferogram (*a* and *b*).



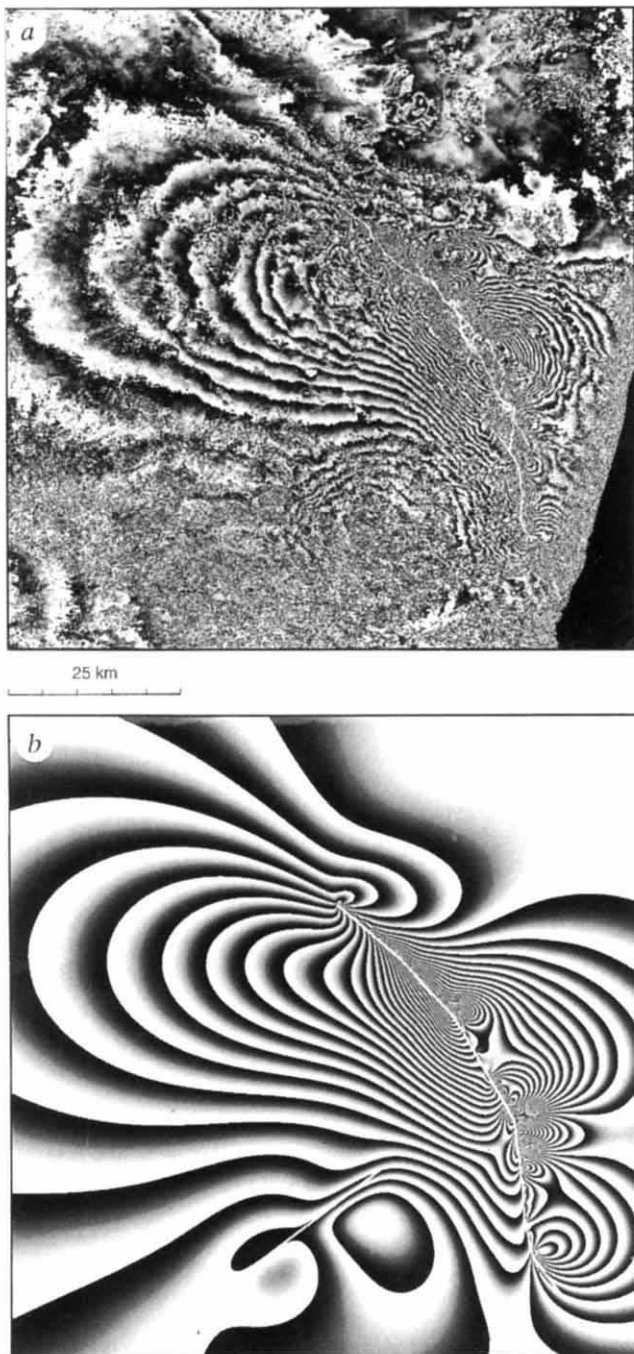


FIG. 3 *a*, Detail of the earthquake area showing the same interferogram as in Fig. 2 and *b* a synthetic interferogram calculated with an elastic half-space dislocation model as described in the text. One cycle of gray shading represents 28 mm of change in the range, as in Fig. 2*a* and *b*. White segments depict the fault geometry as mapped in the field¹¹ (*a*), and as used in the model (*b*). Both images cover the 90 by 110 km area outlined by the medium rectangle in Fig. 1. The observed (*a*) and modelled (*b*) fringe patterns differ mostly in the short-wavelength features near the rupture zone. These local concentrations of strain occur at the ends of fault segments. They are highly sensitive to the detailed geometry of the fault (*a*) and poorly explained by the simple geometry adopted in the model (*b*). The band of incoherence in the rupture zone (*a*) is delimited in many places by two parallel fault splays. These areas are also affected by intense secondary faulting, especially along the northern and southern parts of the Homestead Valley fault¹¹. The loss of correlation might be explained by a large displacement gradient imposed by slip on these secondary faults and possibly rotation of blocks between them.

interferogram by assuming that the observed change in range is due only to horizontal right-lateral strike slip on a plane trending N20°W. The estimated slip (see Fig. 4*b* and associated caption) agrees in magnitude and general distribution with the field observations¹¹.

The Landers earthquake, because of its large and clear surface rupture, provides a positive validation of the use of radar interferometry for measuring co-seismic displacements. Changes in range have been estimated under realistic conditions including actual topography and unexceptional orbits. The resulting map of the displacement field is unprecedented in its combination of precision (34 mm) and dense spatial sampling (100 m per pixel). These attributes could be refined with an improved elevation model and a more precise orbital calculation.

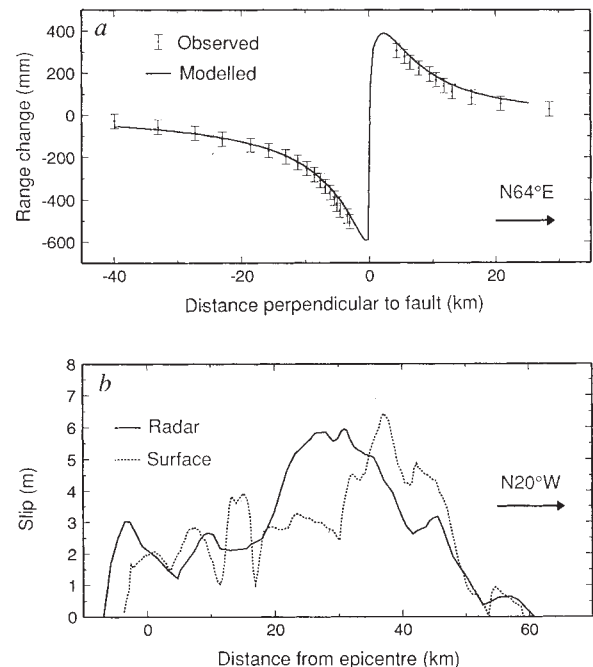


FIG. 4 *a*, Comparison of the co-seismic change in range as observed (points with error bars) and modelled (solid line) along a profile striking N64°E perpendicular to the fault trace at 34.44° N, where the maximum slip was observed¹¹. The observed values are estimated by integrating the phase difference in Fig. 2*a* along the profile. The level of the observed values west and east of the fault is determined by counting fringes along a closed path passing north of the incoherent band in the rupture zone. The origin, with zero change in range, is taken to be where the path crosses the black-to-white fringe contour emanating from the northernmost part of Fig. 2*a*, where we assume no co-seismic displacement. *b*, Comparison of slip distribution along the fault estimated by integration of interferometric fringes (solid line) and from field measurements¹¹ of surface rupture (dashed line). Both quantities are projected onto a line striking N20°W and distance is reckoned positive to the north of the main shock epicentre (34.20° N, 116.44° W). Fringes are counted along two profiles following the western and eastern edges of the incoherent band, at a distance of 5–10 km from the fault. The fringes are extrapolated across the band of incoherence by projecting them in a direction perpendicular to the N20°W strike of the fault. Horizontal slip is derived from the measured slant-range displacement by assuming purely horizontal, right-lateral strike-slip displacement on the N20°W trend. In this approximation, the ratio between slant-range and horizontal slip is the product $\sin\alpha\sin\phi$, where α is the angle between the fault and the satellite nadir ground track and ϕ is the radar incidence angle. The apparent offset of 10 km between the maxima of the two curves results from the right-angle projection of fringes which appear to intersect the fault at an oblique angle. The interferometric estimate of slip does not show all the short wavelength features of the field estimate because the slip on the fault is inferred by extrapolating slip observed at points away from the fault.

tion. Displacements of 1 cm have been detected for artificial targets^{24,25} and the ultimate precision of the technique is at the millimetre level²⁶. For this shallow earthquake, the range changes measured by radar agree extremely well with both the field observations at the fault and the dislocation model in the intermediate and far fields. The detailed features of the radar fringes near the fault require more sophisticated modelling. For deeper earthquakes associated with little or no surface rupture, radar interferometry will become a powerful tool for measuring surface displacement in the intermediate and far fields. Unlike surveying techniques, there is no need to install ground stations before the earthquake. To ensure a pre-earthquake observation, one needs only to archive radar images of the potentially seismogenic area each time the satellite passes over it, as ERS-1 currently does once every 35 days. □

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1. Blewitt, G. *et al.* *Nature* **361**, 340–342 (1993).
2. Bock, Y. *et al.* *Nature* **361**, 337–340 (1993).
3. Murray, M. H., Savage, J. C., Lisowski, M. & Gross, W. K. *Geophys. Res. Lett.* **20**, 623–626 (1993).
4. Graham, L. C. *Proc. Inst. elect. electron. Engrs* **62**, 763–768 (1974).
5. Zebker, H. & Goldstein, R. J. *Geophys. Res.* **91**, 4993–5001 (1986).

6. Gabriel, A. K. & Goldstein, R. M. *Int. J. Remote Sensing* **9**, 857–872 (1988).
7. Li, F. K. & Goldstein, R. M. *IEEE Trans. Geosci. Remote Sensing* **28**, 88–97 (1990).
8. Massonnet, D. *Proc. int. Geophys. appl. Remote Sensing Symp.* **2**, 1431–1434 (1990).
9. Massonnet, D. *Etude de Principe d'une Détection de Mouvements Tectoniques par Radar* Internal memo No. 326 (Centre National d'Etudes Spatiales, Toulouse, 1985).
10. Gabriel, A. K., Goldstein, R. M. & Zebker, H. A. J. *Geophys. Res.* **94**, 9183–9191 (1989).
11. Sieh, K. *et al.* *Science* **260**, 171–176 (1993).
12. Crippen, R. E. *Episodes* **15**, 56–61 (1992).
13. Crippen, R. E. & Blom, R. G. *Eos (Supplement, 27 Oct.)* **73**, 364 (1992).
14. Kanamori, H., Thio, H.-K., Dreger, D. & Hauksson, E. *Geophys. Res. Lett.* **19**, 2267–2270 (1992).
15. Okada, Y. *Bull. seism. Soc. Am.* **75**, 1135–1154 (1985).
16. Shen, Z., Jackson, D., Feng, Y., Kim, M. & Cline, M. *Bull. seism. Soc. Am.* (submitted).
17. Jaume, S. C. & Sykes, L. R. *Science* **258**, 1325–1328 (1992).
18. Stein, R. S., King, G. L. P. & Lin, J. *Science* **258**, 1328–1332 (1992).
19. Hill, D. P. *et al.* *Science* **260**, 1617–1623 (1993).
20. Massonnet, D. & Rabaute, T. *IEEE Trans. Geosci. Remote Sensing* **31**, 455–464 (1993).
21. Massonnet, D., Rossi, M. & Adragna, F. Paper presented at PRISME, CEOS workshop on SAR calibration, Oberpfaffenhofen, Germany, October 9–11 (1991).
22. Elssal, A. A. & Caruso, V. M. *USGS Digital Cartographic Data Standards: Digital Elevation Models* (National Cartographic Information Center, Reston, 1984).
23. Zebker, H. A. & Villasenor, J. *IEEE Trans. Geosci. Remote Sensing* **30**, 950–959 (1992).
24. Cafforio, C., Prati, C. & Rocca, F. *IEEE Trans. aero. Elect. Syst.* **27**, 194–207 (1991).
25. FRINGE SAR Interferometry Working Group, *Proceedings of First Workshop*, 12 Oct. 1992, Frascati, Italy (1992).
26. Gray, A. L. & Farris-Manning, P. J. *IEEE Trans. Geosci. Remote Sensing* **31**, 180–191 (1993).

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The tropics as a source of evolutionary novelty through geological time

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SPATIAL and temporal variations in biological diversity can be shaped by a variety of dynamical interactions between origination and extinction^{1–3}. For this reason, the evolutionary basis of the latitudinal diversity gradient—with the tropics extraordinarily rich in species, higher taxa and evolutionary novelties—has been much debated^{4–8}. High origination rates with the tropics operating as a diversity pump^{8–11}, low extinction rates with the tropics operating as a diversity accumulator^{12–15}, or some combination of the two^{16–18}, have all been proposed to explain the wealth of higher taxa and morphological variety in low latitudes. Few historical data have been available, however, to test directly whether the tropics are 'a cradle or a museum'^{15,19}. A new palaeontological analysis of post-Palaeozoic marine orders shows significantly more first appearances in tropical waters, whether defined latitudinally or biogeographically, than expected from sampling alone. This provides direct evidence that tropical regions have been a major source of evolutionary novelty, and not simply a refuge that accumulated diversity owing to low extinction rates.

Forty-two orders of benthic marine invertebrates have appeared since the beginning of the Mesozoic^{20–22}, and the age, environment, and location of the oldest known members are documented²⁰. These data are difficult to evaluate, however, because palaeontological sampling is severely biased geographically, with maximum density in north temperate latitudes^{23–25}. First occurrences of the 26 orders considered to have good preservation potential²⁰ were restored to their original palaeolatitudes, grouped in 10° belts (Fig. 1a), and compared to two null hypotheses that quantify different aspects of sampling bias:

H₀1: Observed first appearances are determined primarily by preservational factors. This distribution (Fig. 1c) was based on the first occurrences of orders whose members are only lightly mineralized or easily disarticulate into seldom identified fragments. These records of poorly preserved orders are probably

dictated more by vagaries of sampling and preservation than by true biogeographic history, and thus provide one measure of the distribution or discovery rate of deposits yielding especially rich biotas²⁰.

H₀2: Observed first appearances are determined primarily by sampling intensity. This distribution (Fig. 1d) was based on all published records of echinoid species (excluding isolated spines) from the beginning of the Triassic to the Bajocian Stage of the Middle Jurassic, an interval that encompasses over half of the ordinal originations²⁶. Echinoids are relatively common post-Palaeozoic fossils that have attracted considerable palaeontological attention over the past 200 yr, so that the geographic pattern of their individual records provides a general measure of sampling intensity across latitude. Because many of the well-preserved orders²⁰ are in Phylum Echinodermata, echinoids are probably a more appropriate taphonomic control²⁰ than molluscs or other taxa.

First occurrences of the well preserved orders (Fig. 1a, b) are significantly more frequent in tropical seas relative to either of the null hypotheses (Fig. 1c, d). The Early Triassic is especially poorly sampled and poorly understood biogeographically^{20,27}, but results remain significant even when originations in this interval are excluded.

Adjustment of the well preserved ordinal occurrences using H₀1 and H₀2 datasets (Fig. 1e, f) gives a general picture of how far the well preserved orders diverge from null expectations. These can only be very approximate corrections but serve to illustrate, for example, that so few poorly preserved orders and species records occur at 0–20° palaeolatitude that even the low number of well preserved orders appearing there exceeds that expected from sampling. More generally, the relatively wide distribution of first occurrences in Fig. 1a and 1b, despite the massive sampling biases revealed in Fig. 1c and 1d, suggests that improved sampling would shift even more ordinal originations into tropical regions. Also, because comparisons are made within each 10° band, the decrease in global surface area with increasing latitude is factored out as a potential bias.

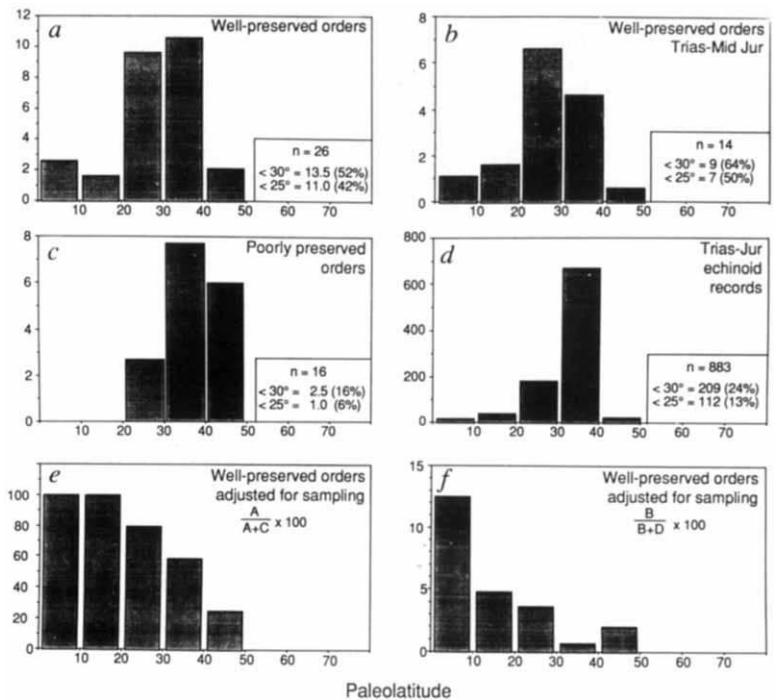
The distribution of reefs and other indicators of tropical conditions has oscillated continually over geological time^{28,29}, so that first occurrences might better be calibrated not to absolute palaeolatitude but to the contemporaneous limits of tropical biotas. Such a biogeographic calibration yields results comparable to the palaeolatitudinal tests (Fig. 2): well preserved orders first occur in tropical settings significantly more frequently than

FIG. 1 First occurrences of post-Palaeozoic marine benthic invertebrate orders, grouped in 10° palaeolatitude belts (N and S plotted on same axis). At this level of resolution (probably the most robust given uncertainties in palaeogeographic reconstructions) the limits of the tropics fall within the $20\text{--}30^\circ$ belt. Use of 5° resolution, with the tropics in the $20\text{--}25^\circ$ belt, does not significantly change the results. Degrees palaeolatitude calculated for the appropriate 5-Myr interval using computer software developed by D. B. Rowley for the Palaeogeographic Atlas Project, University of Chicago. *a*, Well preserved orders, Triassic–Recent; *b*, Triassic–Mid Jurassic (through Bajocian Stage) well preserved orders; *c*, poorly preserved orders, Triassic–Recent (H_0 1, significantly different from *a*, $P < 0.01$, Kolmogorov–Smirnov test on frequency distributions, and $P < 0.001$, G test using either 25° or 30° N and S as an approximate tropical–nontropical boundary); *d*, Triassic–Bajocian echinoid records, calculated as the number of localities from which each species is recorded (H_0 2, significantly different from *b*, $P < 0.025$, Kolmogorov–Smirnov test; $P < 0.005$, G test as above); *e*, well preserved orders from *a*, adjusted for preservation and sampling using the distribution of poorly preserved orders in *c*; *f*, Triassic–Bajocian well preserved orders in *b*, adjusted for sampling using the distribution of echinoid records in *d*. This adjustment procedure effectively factors out potential sampling artefacts arising from the decreasing area of each belt with increasing latitude: well preserved orders are assessed relative to the total number of records within each belt for the H_0 datasets. Despite the larger total area of tropical regions, they are more poorly sampled, so that the proportion of well preserved orders originating in tropical seas is greater than expected. As noted in the text, this does not rule out area differences as a potential biological mechanism for the latitudinal pattern. Orders appearing simultaneously in two adjacent latitudinal belts are scored as 0.5 in each; these are starred and listed here in the lower of the two latitudinal bands. Well preserved orders: $0\text{--}10^\circ$, Pedinoida, Oligopygoida* (contemporaneous records not in adjacent belt but in $21\text{--}30^\circ$), Clypeasteroida; $11\text{--}20^\circ$, Isocrinida*, Pygasteroida; $21\text{--}30^\circ$, Milleporina*, Scleractinia, Cheilostomata, Encrinura, Millericrinura, Hemicidaroida, Phymosomatoida, Holecypoida, Holasteroida*, Spatangoida*; $31\text{--}40^\circ$, Lychniscosida, Helioporacea, Neogastropoda*, Cyrtocrinida*, Bourgueticrinura, Temnopleuroidea, Salenoida (= Calycina), Echinoida, Cassiduloida, Disasteroida; $41\text{--}50^\circ$, Stylasterina. Poorly preserved orders: $21\text{--}30^\circ$, Trichasteropsida, Valvat-

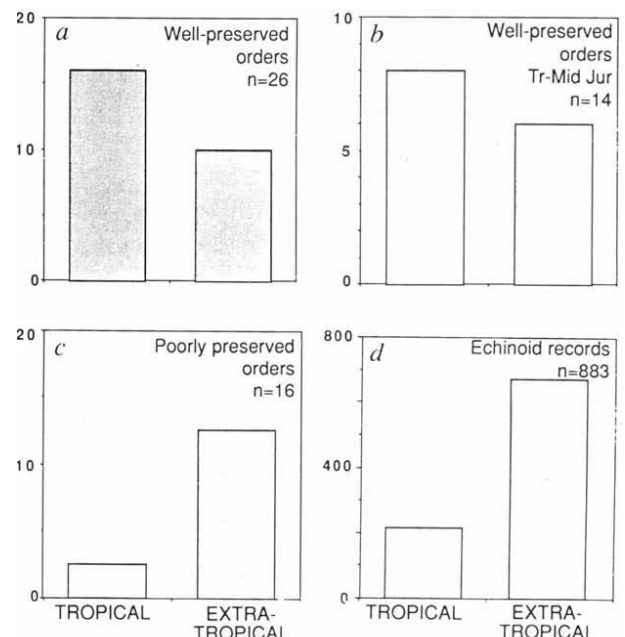
ida, Paxillosida*; $31\text{--}40^\circ$, Stolonifera, Micropygoida, Anaspidea, Notaspidea, Forcipulatida, Notomyoida, Velatida; $41\text{--}50^\circ$, Sacoglossa (= Ascoglossa), Comatulida, Echinothurioida, Diadematoida, Brisingida, Spinulosida. The patterns shown here are robust to alternative taxonomies. They are strengthened if the echinoid superorder Microstomata is used in addition to or instead of Order Cassiduloida²¹; if the crinoid order Bourgueticrinura is subsumed within the Isocrinida³⁰; or if the crinoid family Holocrinidae is considered a stem group to all articulate crinoids rather than part of Order Isocrinida³⁰, with Isocrinida then starting with the mid-Triassic *Laevigatocrinus laevigatus* (Münster) and *Isocrinus tyrolensis* (Laube)³⁰. Inclusion of possible occurrences of two additional poorly preserved orders in the Late Cretaceous of northern Europe²⁰, namely isolated spicules attributed to Homoscleromorpha (Porifera) and borings attributed to Ascothoracica (Arthropoda), would also reinforce the results.

Whether defined palaeolatitudinally or biogeographically, tropical seas have produced more new orders than expected from sampling bias alone. This does not mean that all taxa have tropical origins: the rich molluscan fossil record may indicate a truly extratropical origin for the Order Neogastropoda^{20,30}, and many individual families and genera clearly originated in high latitudes¹⁶. Nor do these results require that ordinal rank *per se* has objective reality across all phyla, or that orders originate with all derived characters in place^{20,21}. They do suggest, however, that novel body plans with the potential to accumulate further derived characters—thereby defining groups likely to be accorded ordinal status—originate most frequently in tropical species.

FIG. 2 First occurrences of orders in tropical and extra-tropical seas, as defined biogeographically on the basis of contemporaneous distributions of tropical indicators such as abundant dasycladalean algae^{39,40} and large benthic foraminifera^{41–43} (to avoid circularity, macroinvertebrate indicators were not used). *a*, Well preserved orders, Triassic–Recent; *b*, poorly preserved orders, Triassic–Recent; *c*, Triassic–Bajocian well preserved orders; *d*, Triassic–Bajocian echinoid records.



ida, Paxillosida*; $31\text{--}40^\circ$, Stolonifera, Micropygoida, Anaspidea, Notaspidea, Forcipulatida, Notomyoida, Velatida; $41\text{--}50^\circ$, Sacoglossa (= Ascoglossa), Comatulida, Echinothurioida, Diadematoida, Brisingida, Spinulosida. The patterns shown here are robust to alternative taxonomies. They are strengthened if the echinoid superorder Microstomata is used in addition to or instead of Order Cassiduloida²¹; if the crinoid order Bourgueticrinura is subsumed within the Isocrinida³⁰; or if the crinoid family Holocrinidae is considered a stem group to all articulate crinoids rather than part of Order Isocrinida³⁰, with Isocrinida then starting with the mid-Triassic *Laevigatocrinus laevigatus* (Münster) and *Isocrinus tyrolensis* (Laube)³⁰. Inclusion of possible occurrences of two additional poorly preserved orders in the Late Cretaceous of northern Europe²⁰, namely isolated spicules attributed to Homoscleromorpha (Porifera) and borings attributed to Ascothoracica (Arthropoda), would also reinforce the results.



Several mechanisms, not necessarily mutually exclusive, might explain the observed pattern. (1) The great species richness of the tropics, both within communities and summed over the immense total area^{5,17,31}, may provide so many evolutionary experiments that the overall probability of ordinal origination is inevitably higher there². Such probabilistic explanations are undermined, however, by bathymetric and temporal disparities between species richness and ordinal origination in the fossil record: orders tend to arise onshore regardless of bathymetric patterns at lower levels²⁰, and tend to appear in the early Palaeozoic and early Mesozoic in advance of prolonged diversity increases and plateaus at lower levels^{6,26,32}. Alternatively, tropical carbonate platforms, which can build out to the shelf edge and thus usurp middle and outer shelf habitats^{33,34}, may bias the tropics on a per-km-of-coastline basis in favour of the shallow-water settings that evidently give rise to most new orders²⁰. (2) The complexity, resource spectrum, or favourableness^{1,11} of the tropics, all variously invoked to explain species richness patterns, may actually confer a higher per species rate of ordinal origination. This seems less likely than the first set of hypotheses because it is unclear why tropical environments should favour greater disparity per speciation event or produce species with greater 'evolutionary potential'. Such arguments focus on background extinction and origination, however, and the tropics are much less stable over geological timescales than previously thought³⁵⁻³⁷. Rebounds of the global marine biota after extinction events are twice as likely to produce new orders than are background processes²⁶, and the potential evolutionary consequences of such upheavals in the tropics, repeatedly disrupting and reassembling these rich communities, are poorly understood. Whatever the ultimate causal mechanism, the present analysis adds a new geographical dimension to the growing evidence^{20,38} that evolutionary novelties do not originate randomly in time and space. □

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- MacArthur, R. H. & Wilson, E. O. *The Theory of Island Biogeography* (Princeton Univ. Press, NJ, 1967).
- Stanley, S. M. *Macroevolution* (Freeman, San Francisco, 1979).
- Sepkoski, J. J. Jr in *The Unity of Evolutionary Biology* (ed. Dudgeon, E. C.) 210-236 (Discordias, Portland, OR, 1991).
- Brown, J. H. & Gibson, A. C. *Biogeography* (Mosby, St Louis, 1983).
- Rosen, B. R. *Helgoländer Meeresunters.* **42**, 269-301 (1988).
- Valentine, J. W. *Evolutionary Paleogeography of the Marine Biosphere* (Prentice-Hall, Englewood Cliffs, NJ, 1973).
- Vermeij, G. J. *Biogeography and Adaptation* (Harvard Univ. Press, Cambridge, MA, 1978).
- Rohde, K. *Oikos* **65**, 514-527 (1992).
- Darlington, P. J. Jr *Zoogeography* (Wiley, New York, 1957).
- Stehli, F. G. et al. *Science* **164**, 947-949 (1969).
- Terborgh, J. *Am. Nat.* **107**, 481-493 (1973).
- Matthew, W. D. *Ann. N.Y. Acad. Sci.* **24**, 171-318 (1915).
- Ehrendorfer, F. *Taxon* **19**, 185-194 (1970).
- Skelton, P. W. et al. in *Major Evolutionary Radiations* (eds Taylor, P. D. & Larwood, G. P.) 91-117 (Clarendon, Oxford, 1991).
- Stebbins, G. L. *Flowering Plants: Evolution above the Species Level* (Harvard Univ. Press, Cambridge, MA, 1974).
- Crame, J. A. *Hist. Biol.* **6**, 37-60 (1992).
- Rosenzweig, M. L. *J. Mamm.* **73**, 715-730 (1992).
- Sohl, J. M. *Ann. Missouri Bot. Gard.* **75**, 117-129 (1988).
- Stenseth, N. C. *Oikos* **43**, 417-420 (1984).
- Jablonski, D. & Bottjer, D. J. in *Major Evolutionary Radiations* (eds Taylor, P. D. & Larwood, G. P.) 15-57 (Clarendon, Oxford, 1991).
- Jablonski, D. & Bottjer, D. J. in *Causes of Evolution* (eds Ross, R. M. & Allmon, W. D.) 21-75 (Univ. Chicago Press, Chicago, 1990).
- Sepkoski, J. J. Jr *Milwaukee Public Mus., Contrib. Biol. Geol.* **83**, 1-156 (1992).
- Simpson, G. G. in *Evolution After Darwin I* (ed. Tax, S.) 117-180 (Univ. Chicago Press, Chicago, 1960).
- Van Valen, L. *Science* **166**, 1656-1658 (1969).
- Raup, D. M. *Paleobiology* **2**, 279-288 (1976).
- Erwin, D. H., Valentine, J. W. & Sepkoski, J. J. Jr *Evolution* **41**, 1177-1186 (1987).
- Erwin, D. H. *Rev. Ecol. Syst.* **21**, 69-91 (1990).
- Ziegler, A. M. et al. in *Fossils and Climate* (ed. Brechley, P. J.) 3-25 (Wiley, Chichester, 1984).
- Crowley, T. J. & North, G. R. *Paleoclimatology* (Oxford Univ. Press, New York, 1991).
- Sohl, N. F. *J. Paleontol.* **61**, 1085-1111 (1987).
- Schopf, T. J. M. et al. in *Marine Organisms: Genetics, Ecology and Evolution* (eds Beardmore, J. A. & Battaglia, B.) 365-386 (Plenum, New York, 1978).
- Signor, P. W. *Am. Rev. Ecol. Syst.* **21**, 509-539 (1990).
- Smith, S. V. *Nature* **273**, 225-226 (1978).
- Sellwood, B. W. in *Sedimentary Environments and Faunas* 2nd edn (ed. Reading, H. G.) 283-342 (Blackwell, Oxford, 1986).
- Colinvaux, P. *Quat. Sci. Rev.* **6**, 93-114 (1987).
- Paulay, G. *Paleobiology* **16**, 415-434 (1990).
- Jablonski, D. *Science* **253**, 754-757 (1991).
- Jablonski, D. & Bottjer, D. J. in *Evolutionary Innovations* (ed. Nitecki, M. H.) 253-288 (Univ. Chicago Press, Chicago, 1990).

- Elliott, G. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **32**, 341-385 (1981).
- Flügel, E. in *Calcareous Algae and Stromatolites* (ed. Riding, R.) 481-503 (Springer, Berlin, 1991).
- Pélissier, T. et al. *Bull. Soc. Géol. France Sér. 7* **24**, 1069-1076 (1982).
- Bassoullet, J.-P. et al. *Bull. Soc. Géol. France Sér. 8* **5**, 699-713 (1985).
- Fleury, J.-J. *Bull. Soc. Géol. France Sér. 8* **5**, 757-770 (1985).

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Stratification of ON and OFF ganglion cell dendrites depends on glutamate-mediated afferent activity in the developing retina

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A FUNDAMENTAL attribute of the vertebrate visual system is the segregation of ON and OFF pathways signalling increments and decrements of light¹⁻⁴. In the mature retina, dendrites of ON- and OFF-centre retinal ganglion cells (RGCs) stratify in different sublaminae of the inner plexiform layer (IPL), and are differentially innervated by two types of bipolar cells which depolarize and hyperpolarize on exposure to light⁵⁻¹⁰. This stratification of ON and OFF RGCs is achieved by the gradual restriction of their dendrites which ramify throughout the IPL early in development¹¹⁻¹⁴. The factors underlying this regressive event are unknown. Dendritic stratification occurs around the time that bipolar cells form synapses in the IPL^{15,16}, which raises the possibility that synaptic activity is involved in this process. Here we test this hypothesis by treating the developing cat retina with the glutamate analogue 2-amino-4-phosphonobutyric acid (APB), which hyperpolarizes ON cone bipolar and rod bipolar cells, thereby preventing their release of glutamate¹⁷⁻¹⁹. We report that intraocular injection of APB during the period when dendritic stratification normally occurs prevents the formation of structurally segregated ON and OFF retinal pathways. These results provide evidence that glutamate-mediated afferent activity regulates the remodelling of RGC dendrites during development.

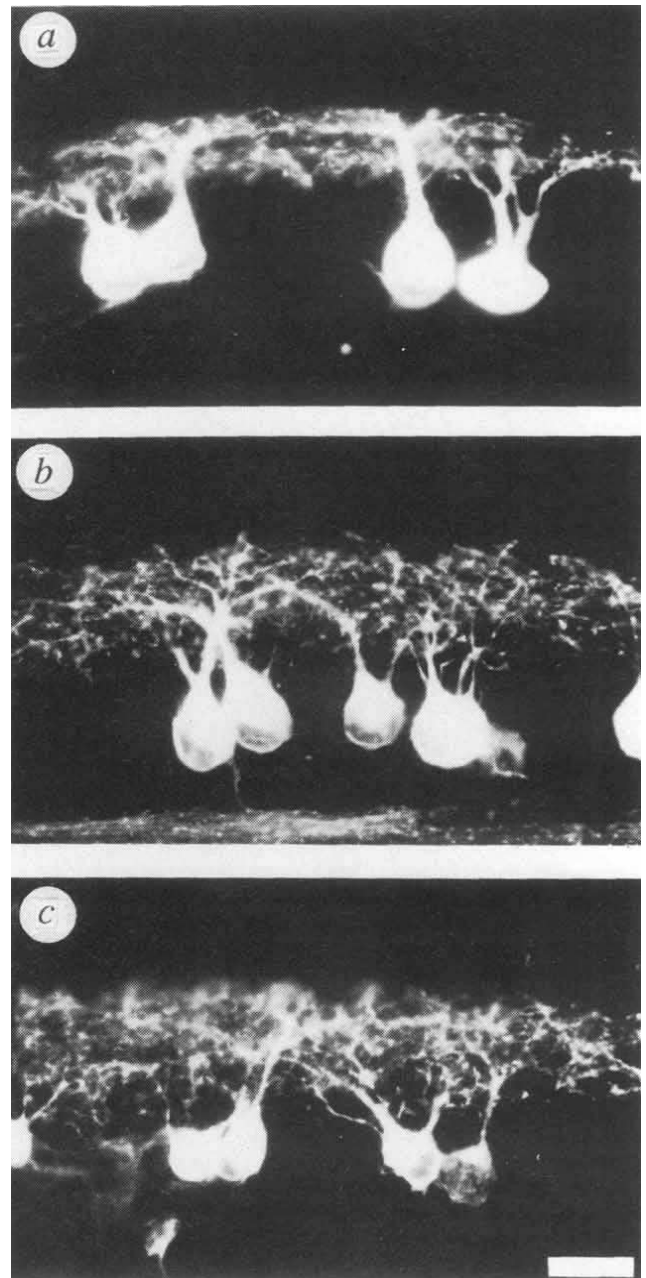
The central region of the cat retina contains the highest density of beta cells which can be readily identified on the basis of soma size and dendritic morphology²⁰. As can be seen in Fig. 1a, the primary dendrites of these neurons stratify either proximal (ON-centre) or distal (OFF-centre) to the soma. In newborn animals, and in those treated with APB, the dendritic morphology of many of these neurons is strikingly different, such that the primary dendrites are multistratified, ramifying both proximal and distal to the soma (Fig. 1b and c).

We first determined the incidence of multistratified ganglion cells in postnatal-day-2 (P2) retinae, the age at which APB injections were initiated. Previous studies have suggested that stratification of cat retinal ganglion cells begins in the central retina before birth and is completed at the periphery several weeks later¹⁶. At P2 a substantial proportion of the dendrites of ganglion cells in the central retina (42.7% of 389 cells) were still multistratified (Fig. 1c). These data provide a baseline for comparing the incidence of multistratified cells in normal and APB-treated retinae of older animals.

We next examined dendritic morphology of beta cells in APB-treated and control retinae. This revealed that the proportion of multistratified cells normally decreases quite markedly by P8, with a gradual reduction occurring during the next 5 days. At

FIG. 1 Light micrographs of Dil (1,1'-diocetadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate) labelled retinal ganglion cells from transverse sections of the central region of *a*, a normal P10 retina, and *b*, an APB-treated retina of a P10 cat, and *c*, a normal P2 retina. In *a*, note the distinct appearance of two tiers of dendrites within the IPL and the stratification pattern of ON (proximal to the cell body) and OFF (distal to the cell body) retinal ganglion cell dendrites. In *b*, note the multistratified appearance of retinal ganglion cell dendrites within the IPL after APB treatment. In *c*, note that at P2, when APB treatment was initiated, the retinal ganglion cell dendrites are multistratified and the appearance of the IPL is like that in *b*. Scale bar, 20 microns.

METHODS. Protocols for animal use were approved by the Animal Care and Use Administrative Advisory Committee of the University of California, Davis, and were in compliance with NIH standards. Animals were premedicated with atropine and anaesthetized with halothane. Intravitreal injections of 10 μ l containing 0.092 mg APB diluted in sterile water were made with a Hamilton syringe. Injections of APB were made daily into one eye of six cats beginning at P2/3. The other eye was untreated ($n=4$) or injected with sterile saline ($n=2$). Pairs of animals were killed at P8, 10 or 13 within 48 h of the last injection. After APB treatment was completed, animals were deeply anaesthetized with pentobarbital, exsanguinated by perfusion with 0.9% saline, followed with a 4% paraformaldehyde fixative. The eyes were removed intact from the cranium and placed for at least one week in the fixative before the optic nerve stumps were implanted with crystals of the lipophilic tracer Dil. After a 7–10-week period to allow for passive diffusion of the tracer, whole retinæ were dissected and sampled by taking circular punches within a 5-mm radius of the area centralis. This tissue was subsequently cross-sectioned for analysis. Some whole-mounted retinæ were analysed in pilot studies, but transverse sections of the retina allowed for a more accurate assessment of stratification within the IPL. Retinal punches were embedded in agar, sectioned at a 40 μ m thickness on a vibratome, mounted onto gelatinized slides and coverslipped with Krystallon. The primary dendrites of Dil-labelled beta cells were examined and identified as branching within one (unistratified) or more than one (multistratified) sublamina of the IPL.



P13 the incidence of such cells is about 12%, indicating that the stratification process is nearing completion in the central region of the retina. In contrast, APB-treated retinæ are characterized by a much higher percentage of multistratified beta cells (Fig. 1*b*). Figure 2 shows that the incidence of multistratified cells in the APB-treated retinæ, unlike the normal case, does not decrease appreciably over the developmental period studied. At P8 there were nearly twice as many multistratified cells in APB-treated retinæ as there were in normal retinæ (APB-treated: 38.8%; normal: 20.7%). By P13 there were almost three times the number of multistratified cells in the APB-treated retinæ as there were in normal retinæ (APB-treated: 34.1%; normal: 12.2%). Overall, there was only a small decrease in the incidence of multistratified cells from that observed at P2, the age when APB treatment was initiated. At the same time, other morphological characteristics of ganglion cells (density, somal sizes, extent and branching of dendrites) in APB-treated retinæ appeared normal. These findings indicate that APB treatment specifically disrupts the normal course of dendritic stratification in developing retinal ganglion cells.

In the adult cat ON- and OFF-centre ganglion cells are characterized by a regular distribution pattern, with the dendrites of each subclass uniformly tiling the retina. These independent

mosaics are superimposed across the retinal surface so that ON and OFF cells of the same class are often found in pairs²⁰. To determine whether APB treatment selectively disrupts stratification of the ON or OFF pathway, we analysed the composition of paired beta cells that contained at least one multistratified member. Such a paired cell analysis minimized the possibility of a sampling bias towards either ON or OFF RGCs. As shown in Table 1, there was a nearly equal incidence of ON/multistratified, OFF/multistratified, as well as pairs of multistratified cells. These proportions were not significantly different in normal and treated retinæ (G -value for goodness of fit was not significantly different, $0.250 > P > 0.100$), indicating that APB treatment disrupted the stratification of both the ON and OFF ganglion cells. This implies that the identity of ON- and OFF-centre ganglion cells is not simply determined by the activity of bipolar cells alone.

The disruption of dendritic stratification of both ON and OFF ganglion cells most likely reflects the fact that rod bipolar cells,

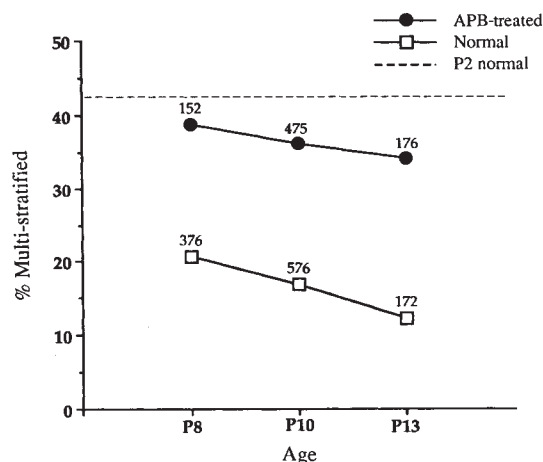


FIG. 2 A comparison of the incidence of multistratified retinal ganglion cells in normal and APB-treated retinas at three postnatal ages. The dotted line denotes the incidence of multistratified cells at P2, the day when APB treatment was started. Two normal and two APB-treated retinas were analysed at each age. The values listed above the data points represent the number of retinal ganglion cells analysed. A statistical analysis of the difference of the frequency of multistratified cells in normal and APB-treated retinas showed that at all ages there were significantly more multistratified cells in APB-treated than in normal retinas (G-test for goodness of fit; all G values significant at the $P < 0.001$ level). There were no significant differences between normal control and vehicle control retinas. Thus comparisons were made between the APB-treated and normal or vehicle control retinas in the same animal. Six of twelve retinas were analysed without the observer knowing whether they were 'treated' or 'control'. There was little variability in the number of multistratified cells after APB treatment between animals at either P8 or P13. At P10, after APB treatment, one of three animals contained fewer multistratified cells (22%) than all the other treated retinas. The advanced state of stratification of ganglion cells in the peripheral untreated retina (50% fewer multistratified cells than in other untreated retinas of the same age) indicated that the retina of this animal was more mature than those of the other P10 animals. This animal was therefore excluded from our study. Retinal ganglion cells in both normal and APB-treated whole-mounted retinas were analysed in pilot studies. Other than the stratification of their dendrites, qualitative observations of cell and dendritic morphology revealed no differences between normal and APB-treated retinas.

which provide indirect inputs to both types of RGCs, are APB-sensitive¹⁹. Rod bipolars terminate on AII amacrine cells, and these cells make a gap junction contact with ON cone bipolar cells and a conventional synapse with OFF ganglion cells²¹⁻²⁴. Thus, a prolonged hyperpolarization of rod bipolar cells by APB would markedly attenuate activity of afferent inputs to all RGCs. Indeed, under scotopic conditions, application of APB completely eliminates visual responses in ON as well as OFF RGCs²⁵. ON cone bipolar cells are also known to be APB-sensitive, but not OFF cone bipolars¹⁷. In the rod-dominated cat retina, the normal functioning of OFF cone bipolars apparently was not sufficient to induce retraction of dendrites of OFF RGCs in the APB-treated retinas. This reasoning assumes that the effects of APB treatment on retinal transmission are equivalent in neonatal and adult animals; further study is required to clarify this point.

The role of activity in the development of the visual system^{26,27}, even before birth²⁸, is well-established. In particular, refinements of retinal ganglion cell terminal arbors can be disrupted by tetrodotoxin (TTX), by NMDA (N-methyl-D-aspartate) antagonists presumed to be acting on receptors of retinorecipient neurons, and by early monocular deprivation²⁸⁻³¹. In contrast, the stratification of RGC dendrites is not altered by intraocular TTX application^{32,33} or by visual

TABLE 1 Composition of retinal ganglion cell pairs

	OFF/Multi	ON/Multi	Multi/Multi
Normal (N = 129)	27.1% (35)	41.1% (53)	31.8% (41)
APB (N = 213)	32.4% (69)	36.1% (77)	31.5% (67)

The composition of retinal ganglion cell pairs across all ages in normal and APB treated retinas, which contained at least one multistratified member. The data for the P8, P10 and P13 retinas are pooled as no age-related effects were evident. There was no significant difference in the composition of retinal ganglion cell pairs between the normal and treated retinas, indicating that APB treatment affected the stratification of both the ON and OFF ganglion cells.

deprivation^{34,35}. Our results thus provide the first demonstration of interference with this regressive event in the developing retina. As dendritic stratification is markedly perturbed after APB treatment, but develops normally when RGC action potentials are blocked by TTX, this implies that the normal restriction of dendritic processes within the sublaminae of the IPL is dependent on synaptic activity. One possibility is that synaptic contacts within the developing IPL are confined to either the distal or proximal processes of initially multistratified ganglion cells. If this were the case, an activity-mediated afferent signal might tell immature ganglion cells which process to retain and which to retract. Alternatively, afferent activity may simply trigger a program of dendritic restructuring in developing retinal ganglion cells. Because these regressive events begin before birth and are completed during the early neonatal period when photoreceptors are still being generated, visual input would not be expected to play a major role. The relevant afferent signals could be the result of activity intrinsic to the developing retina, involving the release of glutamate by bipolar cells. In addition to the issue of underlying mechanisms, it would be of interest to assess the degree to which the observed disruption of retinal ON and OFF pathways correlates with functional changes, and how this might influence the organization of central visual pathways. □

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- Hartline, H. K. *Am. J. Physiol.* **121**, 400-415 (1938).
- Kuffler, S. W. *J. Neurophysiol.* **16**, 37-68 (1953).
- Barlow, H. B. *J. Physiol.* **119**, 69-88 (1953).
- Schiller, P. H. *Trends Neurosci.* **15**, 86-92 (1992).
- Nelson, R. & Kolb, H. *Vision Res.* **23**, 1183-1195 (1983).
- Famiglietti, E. V. Jr & Kolb, H. *Science* **194**, 193-195 (1976).
- Nelson, R., Famiglietti, E. V. Jr & Kolb, H. *J. Neurophysiol.* **41**, 472-483 (1978).
- Wässle, H., Boycott, B. B. & Illing, R. B. *Proc. R. Soc. Lond. B* **212**, 177-195 (1981).
- McGuire, B. A., Stevens, J. K. & Sterling, P. *J. Neurosci.* **6**, 907-918 (1986).
- Wässle, H. & Boycott, B. B. *Physiol. Rev.* **71**, 447-480 (1991).
- Maslim, J., Webster, M. & Stone, J. *J. comp. Neurol.* **254**, 382-402 (1986).
- Maslim, J. & Stone, J. *Dev. Br. Res.* **44**, 87-93 (1988).
- Ramoa, A. S., Campbell, G. & Shatz, C. J. *J. Neurosci.* **8**, 4239-4261 (1988).
- Dann, J. F., Buhl, E. H. & Peichl, L. *J. Neurosci.* **8**, 1485-1499 (1988).
- Maslim, J. & Stone, J. *Brain Res.* **373**, 35-48 (1986).
- Maslim, J. & Stone, J. *Dev. Br. Res.* **44**, 87-93 (1988).
- Slaughter, M. M. & Miller, R. F. *Science* **211**, 182-185 (1981).
- Bolz, J., Wässle, H. & Thier, P. *Neurosci.* **12**, 875-885 (1984).
- Müller, F., Wässle, H. & Voigt, T. *J. Neurophysiol.* **59**, 1657-1672 (1988).
- Wässle, H., Boycott, B. B. & Illing, R. B. *Proc. R. Soc. Lond. B* **212**, 177-195 (1981).
- Kolb, H. & Famiglietti, E. V. *Science* **186**, 47-49 (1974).
- Famiglietti, E. V. & Kolb, H. *Brain Res.* **84**, 293-300 (1975).
- McGuire, B. A., Stevens, J. K. & Sterling, P. *J. Neurosci.* **4**, 2920-2938 (1984).
- McGuire, B. A., Stevens, J. K. & Sterling, P. *J. Neurosci.* **6**, 907-918 (1986).
- Wässle, H., Yamashita, M., Greferath, U., Grünert, U. & Müller, F. *Vis. Neurosci.* **7**, 99-112 (1991).
- Movshon, J. A. & van Sluysers, R. C. A. *Rev. Psychol.* **32**, 477-522 (1981).
- Sherman, S. M. & Spear, P. D. *Physiol. Rev.* **62**, 740-855 (1982).
- Shatz, C. J. & Stryker, M. P. *Science* **242**, 87-89 (1988).
- Sretavan, D. W., Shatz, C. J. & Stryker, M. P. *Nature* **336**, 468-471 (1988).
- Shatz, C. J. *Neuron* **5**, 745-756 (1990).
- Hahm, J. O., Langdon, R. B. & Sur, M. *Nature* **351**, 568-570 (1991).
- Wong, R. O. L., Herrmann, K. & Shatz, C. J. *J. Neurobiol.* **22**, 685-697 (1991).
- Dubin, M. W., Stark, L. A. & Archer, S. M. *J. Neurosci.* **6**, 1021-1036 (1986).
- Leventhal, A. G. & Hirsch, H. V. B. *J. Neurosci.* **3**, 332-344 (1983).
- Lau, K. C., So, K. F. & Tay, D. J. *comp. Neurol.* **300**, 583-592 (1990).

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Reversal of long-term potentiation by inhibitors of haem oxygenase

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EVIDENCE that carbon monoxide can serve as an intercellular messenger in brain¹⁻³, a role much like that demonstrated for nitric oxide in various tissues⁴, prompted us to investigate whether carbon monoxide participates in long-term potentiation (LTP), the cellular mechanism that may underlie certain forms of learning and memory. Although LTP is triggered in the postsynaptic neuron, at least some fraction of LTP is expressed presynaptically as an increase in the quantity of neurotransmitter released⁵⁻¹⁴. Thus, a retrograde signal must form the communication link between the postsynaptic site of induction and the presynaptic site of expression. To test whether carbon monoxide might act as a retrograde signal in LTP, we have investigated the effect on LTP of inhibitors of the enzyme haem oxygenase-2, which catalyses the production of carbon monoxide in the brain. We find that these inhibitors prevent the induction of LTP and have no effect on one form of long-term depression. Furthermore, they will reverse LTP that is already established.

We used whole-cell voltage clamp of rodent CA1 pyramidal cells in slices, a method that produces large LTP and has the advantage that the magnitude of LTP is independent of stimulus strength. The haem oxygenase inhibitors used in our experiments were zinc protoporphyrin IX (ZnPP) and zinc deuterioporphyrin IX 2,4-bis glycol (ZnBG)^{15,16}. ZnPP blocks LTP (Fig. 1).

Although whole-cell recording is a very sensitive way to determine the presence or absence of LTP, only a small number of neurons are sampled. For that reason, we have also used conventional field potential recording to examine LTP in 27 control and experimental slices treated with either ZnPP (5 to 15 μ M; $n=6$) or ZnBG (10 μ M; $n=9$). The mean response 30 min post-tetanus was $140 \pm 5.5\%$ (mean $\pm$ s.e.m.) for the control slices and $102 \pm 2.5\%$ for the drug-treated slices. A typical result for a field potential recording experiment appears in Fig. 2, for which no appreciable LTP was elicited in three attempts.

Zn^{2+} ions block *N*-methyl-D-aspartate (NMDA) receptors. We considered, however, that the chelated zinc in ZnPP would be unlikely to have access to the NMDA receptor blocking site and, if it did, it should be ineffective at the concentrations used here¹⁷. Nevertheless, we examined the effect of ZnPP on NMDA-receptor-mediated synaptic currents in three CA1 neurons by blocking the non-NMDA components (10 μ M CNQX) and measuring the size of the NMDA component before and after application of ZnPP (10 or 15 μ M). We detected no appreciable effect (mean $\pm$ s.e.m. = 22.77 ± 2.55 pA before inhibitor, and 22.80 ± 0.90 pA measured after a 15–36-min treatment). In addition, 20 μ M ZnCl_2 did not block LTP ($n=1$).

Long-term depression (LTD)^{18,19}, like LTP, depends on NMDA receptor function; we therefore examined the effect of ZnPP on LTD. LTD was unaffected by ZnPP (Fig. 2). For four control and seven ZnPP-treated slices, the magnitudes of LTD (expressed as decreased response size as a per cent of the baseline response size) were $25 \pm 2.2\%$ and $28 \pm 2.2\%$ (mean $\pm$ s.e.m.) respectively, values that are not significantly different ($p > 0.4$ by *t*-test). In addition, 50 μ M AP5 did block LTD in three slices that were treated with 10 μ M ZnPP and one slice treated with 5 μ M ZnBG (field excitatory postsynaptic potential (e.p.s.p.) slope 30 min after LFS was $100.3 \pm 2.8\%$), showing that the NMDA-receptor-dependent form of LTD was being observed in these experiments. This result is important for two reasons.

FIG. 1 ZnPP prevents LTP: whole cell recording. Peak excitatory post-synaptic current (e.p.s.c.) in CA1 pyramidal cells, expressed as a per cent of the baseline (bsl) value, as a function of time during the experiment for A, a control neuron, and B, a neuron in a slice treated with 10 μ M ZnPP for the time indicated by the line in the figure. Tetanic stimulation was given at the time indicated by the arrowheads. Soma membrane potential was maintained at -70 mV throughout except during the tetanus, when it was set to -30 mV. The insets are sample records of e.p.s.c.; the left inset is taken before the tetanus and the right inset taken ~ 30 min after the tetanus. The calibration bars indicate 20 pA (vertical) and 10 ms (horizontal). Points on the plot represent averages of two or three successive observations. C, Cumulative histograms that specify the relative frequencies of finding LTP of the size specified on the abscissa, measured between 25 and 40 min after the tetanus, for 12 control cells (a) and 8 cells (b) in slices treated with ZnPP (10 μ M for seven slices and 15 μ M for one slice). The ordinate shows the fraction of slices with a post-tetanus response size less than the value given on the abscissa; the average peak e.p.s.c. for the ten minutes at least before the tetanic stimulation is normalized to 100%. The two cumulative histograms are significantly different (two-sided Kolmogorov-Smirnov test; $P < 0.01$).

METHODS. Standard methods were used for preparation of slices and electrophysiological recordings. Briefly, transverse hippocampal slices (350–400 μ m) were obtained from mice (3–7 weeks, C57/Bl-6J; Jackson) or rats (2–6 weeks, Harlan-Sprague-Dawley). A single slice was rested in a submerged recording chamber perfused continuously with artificial cerebrospinal fluid (ACSF) at a rate of ~ 2 ml min^{-1} and at $31.5 \pm 0.5^\circ\text{C}$. The ACSF, equilibrated with 95% O_2 /5% CO_2 , is composed of (in mM): NaCl (120), KCl (3.5), NaH_2PO_4 (1.25), NaHCO_3 (26), MgCl_2 (1.3), CaCl_2 (2.5). Picrotoxin (PCTX, 25–75 μ M) was included in ACSF in most experiments to block GABA-mediated inhibitory transmission. Extracellular field excitatory postsynaptic potentials (f.e.p.s.p.s) were recorded in the apical dendrite layer of CA1 with electrodes (1–2 M Ω) filled with ACSF. Excitatory postsynaptic currents (E.P.S.Cs) were recorded in CA1 pyramidal neurons (CA3 region was usually removed) with the whole-cell patch-clamp mode. Electrodes (3–5 M Ω) were filled with (in mM) caesium gluconate (130), CsCl (5), EGTA (0.5), MgCl_2 (1), Mg-ATP (2), GTP (0.2), NaCl (5), HEPES (10); pH 7.25. Bipolar tungsten stimulating electrodes were used to apply voltages (100- μ s duration) to Schaffer collateral-commissural afferents to evoke f.e.p.s.p.s at 30- to 40-s intervals (2–3 min for the experiment lasting > 2 h) or to evoke E.P.S.Cs at a 10- to 15-s intervals. The tetanus in field potential recording was composed of 5 or 6 bursts of 20 pulses, each with an interpulse interval of 10 ms and an interburst interval of 10 s. The tetanus in whole-cell recording was composed of 3 or 4 bursts with the same frequency and interburst interval as above, while holding membrane potential at -30 mV. The protocol for inducing LTD was to stimulate slices or neurons at 1 Hz for 10 to 15 min. For LTD experiments that used whole-cell recording, the soma membrane potential was maintained at -70 or -80 mV throughout the period of 1 Hz stimulation. Data (filtered at 1 kHz and sampled at 5 kHz) were collected and analysed with programs written by us in QuickBASIC/AxoBASIC. ZnPP was obtained from Aldrich or Porphyrin Products (Utah) and ZnBG from Porphyrin Products. ZnPP dissolved in DMF (*N,N*-dimethylformamide) or DMSO (dimethyl sulphoxide) was freshly prepared and diluted in ACSF to 5–15 μ M (usually 10 μ M) just before use, and perfused (bath) for 15 to 30 min. ZnBG was dissolved in H_2O with a little NaOH and typically applied to the slices for 25–40 min at concentrations of 5–10 μ M. Exposure of ZnPP and ZnBG to bright light was avoided. The final concentration of DMF was 0.05–0.1%, which had no significant effect on normal synaptic transmission and LTP ($n=3$). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; non-NMDA receptor antagonist) and d-APV (D,L-2-amino-5-phosphonopivalic acid; NMDA receptor antagonist) were from Research Biochemicals.

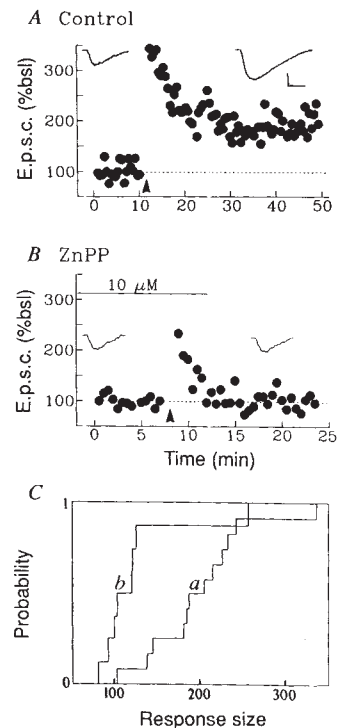
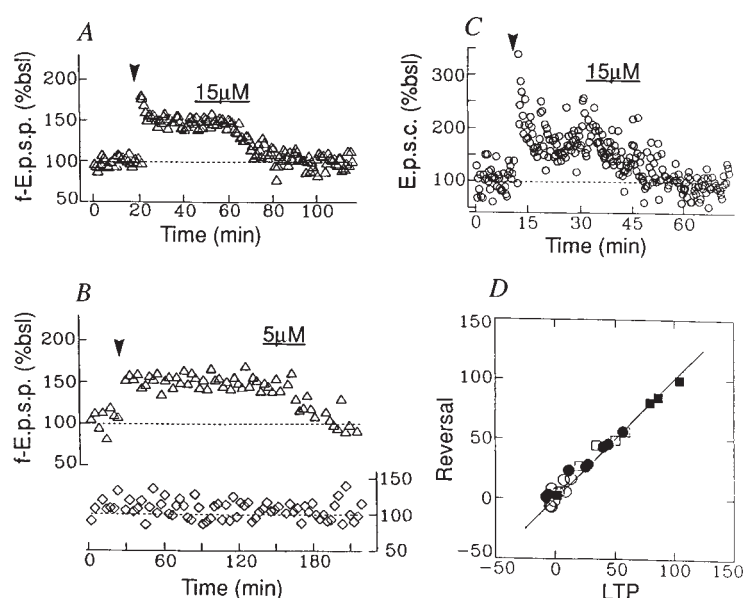


FIG. 3 Elimination of established LTP with ZnPP. **a, b** and **c**, Response size, expressed as a per cent of baseline, as a function of time. A tetanic stimulus was presented at the time indicated by the arrowheads to produce LTP, and ZnPP (15 μ M) or ZnBG (5 μ M) was applied for the durations indicated by the horizontal lines above the data points. Field potential recording was used for **a** and **b**, and whole cell recording for **c**. In **b**, two separate pathways in the slice were used; the data shown at the top are from the pathway that was tetanized and at the bottom they are from the control pathway. In **d**, the amount of LTP (30 min after the tetanus) is plotted against the amount of reversal produced by ZnPP or ZnBG. Squares represent experiments in which only a single pathway was used (open squares for field potentials, $n=5$; filled squares for whole cell recording, $n=4$) and circles represent field potential recording experiments in which two pathways, control and tetanized were used (filled circles for the tetanized pathway and open circles for the control pathway, $n=8$). Each point was determined by measuring the average response size over a period of time (20–40 min pretetanus; 10 to 20 min at a time 30 min after LTP was established; 20 to 40 min after response size stabilized following drug treatment). The magnitude of LTP on the abscissa is expressed as a percentage increase in response size over the baseline, so 0% represents no effect of the tetanus (no LTP) and 100% indicates that the response doubled. The percentage decreases plotted on the ordinate are calculated so that 0% means that the ZnPP or ZnBG produced no change in response size. The reference level for calculating these percentages is such that a 100% LTP and a 100% reversal would return the response size to its starting (pretetanus) level.



First it provides additional evidence that the block of LTP by haem oxygenase inhibitors is not the result of effects on NMDA receptor channels because an independent consequence of NMDA receptor activation, that is, LTD, is still intact in our slices. Second, these experiments demonstrate that the metalloporphyrins do not simply inhibit all forms of long-lasting synaptic plasticity, but rather block one form of plasticity (LTP) selectively. Finally we note that this is, to our knowledge, the first pharmacological treatment that has been described to selectively block LTP without affecting LTD.

LTD can erase LTP, but we are aware of only one pharmacological agent, the complex kinase inhibitor H-7, which has been reported to have a similar effect²⁰. We applied inhibitors of haem oxygenase to slices after various amounts of LTP, ranging between 0 and 205%, had been established, and found that ZnPP seemed to erase the pre-existing LTP (Fig. 3a). Fig. 3b shows that ZnPP removed (or suppressed) LTP in an experiment in which two non-interacting pathways were used, one of which is stimulated by tetanus to produce LTP without affecting the con-

trol pathway. This same result has been obtained in eight dual pathway experiments, two with ZnPP and six with ZnBG (5 or 10 μ M). We have also found that ZnPP reverses LTP under whole-cell recording ($n=4$), a situation in which we can control the membrane potential (Fig. 3c); thus this effect still occurs when the neuron has been dialysed for an hour.

The percentage decrease of response size after application of both ZnPP and ZnBG closely equals the percentage increase produced earlier by the tetanus (Fig. 3d). If the experimentally produced LTP were exactly reversed, the points would lie on the diagonal line in Fig. 3d; depression that exceeds the amount of LTP would give points to the left of the line and failure to eliminate LTP completely would appear as points to the right of the line. The good fit of observed points to the diagonal line supports the conclusion that ZnPP and ZnBG block the expression of different experimentally produced amounts of LTP but have no significant effect on basal synaptic transmission at the drug concentrations we have used. Also haem oxygenase inhibitors have no effect on synaptic strength when transmitter release is increased pharmacologically (data not shown).

We are uncertain whether or not CO plays a role in LTP because the inhibitors may have had effects apart from the inhibition of haem oxygenase^{21,22}. Recent results²³, however, complement our observations and provide further evidence in favour of the CO hypothesis. □

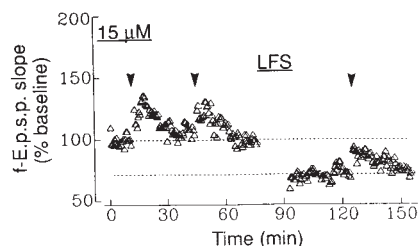


FIG. 2 LTD is unaffected by ZnPP. Plots field e.p.s.p. slope (mV ms^{-1}), expressed as per cent of the baseline value, as a function of time during the experiment. The slice was treated with 15 μ M ZnPP at the time indicated by the line at top left. Each arrowhead indicates the time of tetanic stimulus. During the period labelled LFS, the slice was subjected to 1 per second stimulation to produce LTD. Note that the LTD could not be reversed by the last tetanic stimulation. This same result was obtained from four slices treated with ZnPP (5–15 μ M), but LTD was readily reversed by tetanic stimulation in four control slices.

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- Verma, A. D. J., Glatt, C. E., Ronnett, G. V. & Snyder, S. H. *Science* **259**, 381–384 (1993).
- Marks, G. S., Brien, J. F., Nakatsu, K. & McLaughlin, B. E. *Trends pharmac. Sci.* **12**, 185–188 (1991).
- Ewing, J. F. & Maines, M. D. *Molec. cell. Neurosci.* **3**, 559–570 (1992).
- Culotta, E. & Koshland, D. E. Jr *Science* **258**, 1862 (1992).
- Bliss, T. V. P., Douglas, R. M., Errington, M. L. & Lynch, M. A. *J. Physiol.* **377**, 391–418 (1986).
- Voronin, L. L. in *Synaptic Plasticity in the Hippocampus* (eds Haas, H. L. & Buzsaki, G.) 27–30 (Springer, Berlin, 1988).
- Malinow, R. & Tsien, R. W. *Nature* **346**, 177–180 (1990).
- Bekkers, J. M. & Stevens, C. F. *Nature* **346**, 724–729 (1990).
- Baskys, A., Carlen, P. L. & Wojtowicz, J. M. *Neurosci. Lett.* **127**, 169–172 (1991).
- Malinow, R. *Science* **252**, 722–724 (1991).
- Malgaroli, A. & Tsien, R. W. *Nature* **357**, 134–139 (1992).
- Kullmann, D. M. & Nicoll, R. A. *Nature* **357**, 240–244 (1992).
- Larkman, A., Hannay, T., Stratford, K. & Jack, J. *Nature* **360**, 70–73 (1992).
- Liao, D., Jones, A. & Malinow, R. *Neuron* **9**, 1089–1097 (1992).
- Maines, M. D. *Biochim. Biophys. Acta* **673**, 339–350 (1981).

16. Vreman, H. J., Lee, O. K. & Stevenson, D. K. *Med. Sci.* **302**, 335–341 (1991).
17. Mayer, M. L. & Vyklicky, L. Jr *J. Physiol.* **415**, 351–365 (1989).
18. Dudek, S. M. & Bear, M. F. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4363–4367 (1992).
19. Mulkey, R. M. & Malenka, R. C. *Neuron* **9**, 967–975 (1992).
20. Malinow, R., Madison, D. V. & Tsien, R. W. *Nature* **335**, 820–824 (1988).
21. Bloomer, J. R., Reuter, R. J. & Morton, K. O. *Gastroenterology* **85**, 663–668 (1983).
22. Ignarro, L. J., Bailot, B. & Wood, K. S. *J. Biol. Chem.* **259**, 6201–6207 (1984).
23. Zhou, M., Small, S. A., Kandel, E. A. & Hawkins, R. D. *Science* (in the press).

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Protective effects of oligosaccharides in P-selectin-dependent lung injury

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NEUTROPHIL recruitment into tissues is a multistep process involving sequential engagement of adhesion molecules, including selectins (E,P,L), which are reactive with oligosaccharides, and the family of $\beta 2$ integrins which are reactive with endothelial intercellular adhesion molecules^{1–3}. These processes result in the initial rolling of leukocytes along the endothelial surfaces, followed by the firm attachment of leukocytes to the endothelium. The intravenous infusion of cobra venom factor into rats results in acute lung injury that is neutrophil-dependent, oxygen radical mediated and P-selectin-dependent^{4,5}. Here we report that infusion of sialyl-Lewis X, a ligand for P-selectin^{6–9}, dramatically reduced lung injury and diminished the tissue accumulation of neutrophils, whereas irrelevant oligosaccharides had no such effects. These results suggest that sialyl-Lewis X carbohydrates may be used as a new strategy for anti-inflammatory therapy.

We used several sialyl-Lewis X (SLX)-related oligosaccharides, the structures of which are shown in Fig. 1. The ability of the SLX reducing sugar (SLX-OH) to reduce neutrophil accumulation and diminish lung injury after infusion of cobra

venom factor (CVF) is shown in Fig. 2. These effects contrast with the lack of protection when the non-fucosylated form of SLX-OH, sialyl-N-acetylactosamine (SLN-OH), was used (Fig. 2). The positive controls (injected intravenously with CVF in phosphate buffered (pH 7.4) saline, PBS) showed a sixfold increase in lung permeability (Fig. 2a), a fivefold increase in haemorrhage (b) and a fivefold increase in lung myeloperoxidase (MPO) content. When 200 μ g SLN-OH (a control carbohydrate which does not support P-selectin-mediated adhesion⁸) was infused immediately before injection of CVF, the permeability, haemorrhage and MPO values were unaffected, whereas treatment with SLX-OH reduced the permeability value by 43% ($P=0.001$), haemorrhage by 41% ($P=0.004$) and MPO content by 35% ($P=0.006$).

Dose-response relationships were evaluated with two other SLX analogues with similar potency: SLX-tetrasaccharide (SLX-tetra) and an SLX-pentasaccharide (SLX-penta). Both SLX analogues showed similar potency with maximal inhibition achieved at a dose of 200 μ g (Fig. 3). Over a dose range of oligosaccharide of 50–500 μ g, treatment with SLX-penta reduced the permeability values by as much as 67% (Fig. 3a), haemorrhage by as much as 47% (b), and lung MPO content by as much as 49% (c). These data indicate that three different analogues of SLX have significant protective effects against CVF-induced lung injury, that these protective effects are dose-dependent and plateau at 200 μ g, and that the protective effects correlate with reduced neutrophil content in the lung, as defined by lung content of MPO. Note that the protective effects of the relevant oligosaccharides (this report) quantitatively parallel the protective effects of murine monoclonal anti-P-selectin in the CVF model of lung injury⁵.

As indicated above, infusion of 200 μ g SLX-pentasaccharide 5 min before injection of CVF reduced permeability and haemorrhage by 67% ($P<0.01$) and 47% ($P<0.01$), respectively. When the intravenous infusion of SLX-penta was delayed until 5 min after infusion of CVF, permeability and haemorrhage (as compared with values in SLN-treated rats) were reduced by 46% ($P=0.034$) and 38% ($P=0.013$), respectively, whereas when the oligosaccharide was infused 15 min after CVF, the permeability and haemorrhage parameters fell by 36% ($P=0.007$) and 25% ($P=0.014$), respectively. Thus the protective effects of SLX-penta in the CVF model of lung injury are time-dependent in relation to the infusion of CVF.

Rats were infused intravenously with PBS, 200 μ g SLX-penta or its non-fucosylated analogue (SLN-penta) before treatment with CVF and the lungs were prepared for light microscopy 30 min later. Figure 4 shows the expected evidence of intra-alveolar haemorrhage and neutrophil accumulation along endothelial surfaces, whereas in the SLX-penta-treated animals, there was

Sialyl-Lewis X (SLX)

$\text{NeuAc}\alpha 2,3\text{Gal}\beta 1,4$ GlcNAc-R $\text{Fuc}\beta 1,3$	Abbreviation	R group
	SLX-OH	OH
	SLX-tetra	$\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$
	SLX-penta	$\beta 1,3\text{Gal}\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$

FIG. 1 Synthetic sialyl-Lewis X (SLX) oligosaccharides and non-fucosylated analogues (SLN) used as inhibitors of CVF-induced lung injury. Oligosaccharides were synthesized by a combined chemical and enzymatic approach^{13,14} (S.D. and Z.L.Z., unpublished observations).

Sialyl-N-Acetylactosamine (SLN)

$\text{NeuAc}\alpha 2,3\text{Gal}\beta 1,4\text{GlcNAc-R}$	Abbreviation	R group
	SLN-OH	OH
	SLN-tetra	$\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$
	SLN-penta	$\beta 1,3\text{Gal}\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$

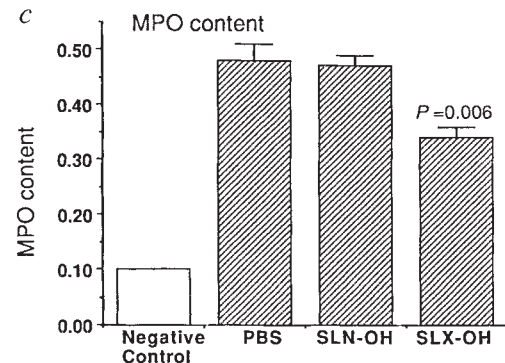
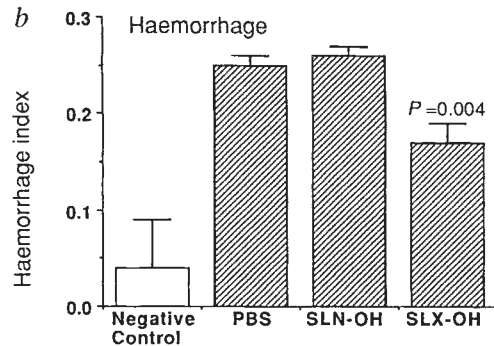
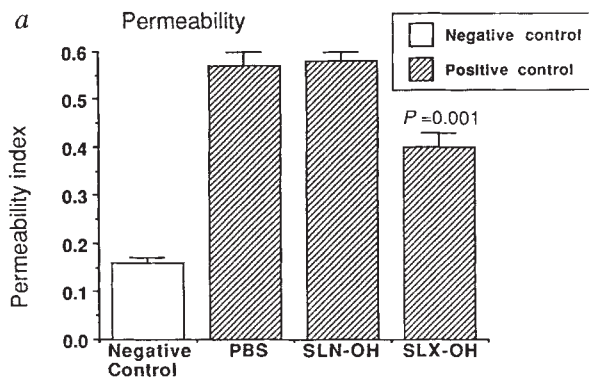


FIG. 2 Lung injury after bolus intravenous infusion of CVF (20 units), as assessed at 30 min by leakage into lung parenchyma of ^{125}I -BSA (a), extravasation of ^{51}Cr -rat red blood cells (RBC) (b), and tissue accumulation of neutrophils (MPO content) (c). Negative control rats (300 g male Long Evans) received 0.5 ml PBS intravenously, whereas other groups received 0.25 ml CVF at time 0 preceded 5 min earlier by 0.25 ml PBS, PBS with 200 μg SLX-OH or PBS with 200 μg non-fucosylated form of SLX-OH (SLN-OH). MPO content was determined as described elsewhere⁵. Protection against injury was calculated by subtracting negative control values from each positive control treatment group and then comparing injury in the CVF group with those CVF groups pretreated with oligosaccharide preparations. For each vertical bar, $n=6$.

much reduced evidence of these changes.

The concentrations of SLX-OH required to mediate the protective effects in the CVF lung injury model are surprisingly low. It can be calculated that the 200 μg dose of SLX in the rat would be rapidly reduced by dilutional changes to less than 10 μg ml^{-1} blood, resulting in a blood concentration of <1 μM . The

ability of SLX preparations to show significant protective effects in the CVF model of acute lung injury in the rat provides further evidence that selectin-dependent adhesive interactions between leukocytes and endothelial cells may be appropriate targets of anti-inflammatory interventions based on the use of oligosaccharide analogues of their ligands.

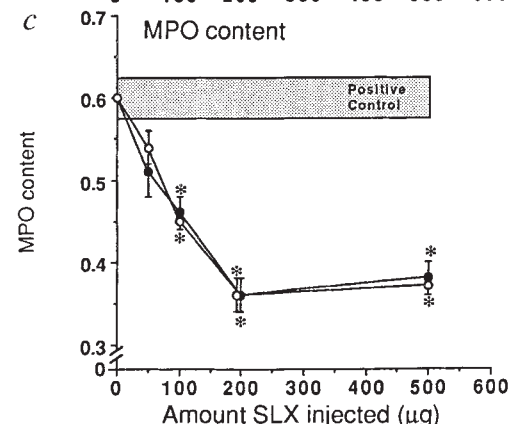
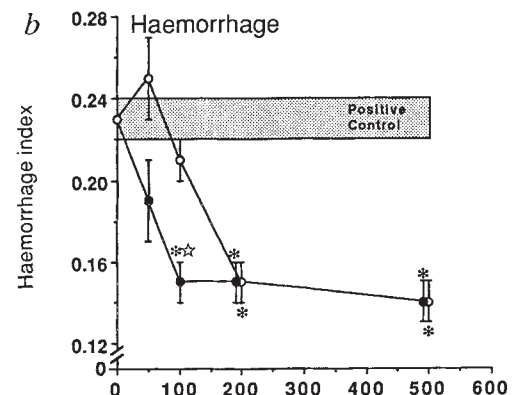
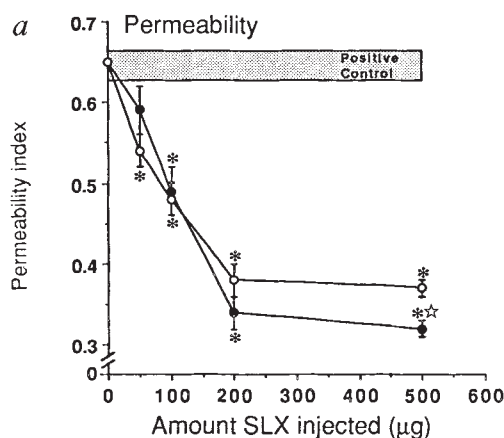


FIG. 3 Dose-response relationships of CVF-infused rats pretreated with varying amounts of tetrasaccharide or pentasaccharide preparations of SLX, used as single intravenous doses (50–500 μg) in 0.25 min before intravenous infusion of CVF. Lung injury parameters of permeability (a), haemorrhage (b) and MPO content (c) were measured according to details described in Fig. 2. The positive control (animals also receiving 200 μg SLN-OH) reference points (mean $\pm$ s.e.m.) are indicated by the horizontal area ('positive control'). For each data point, $n=5$. $\circ$, SLX-tetra; $\bullet$, SLX-penta. * $P<0.01$ (a,c), <0.05 (b) when compared with positive controls; † $P<0.05$ (a), or <0.01 (b) when compared with tetrasaccharide group at same dose level.

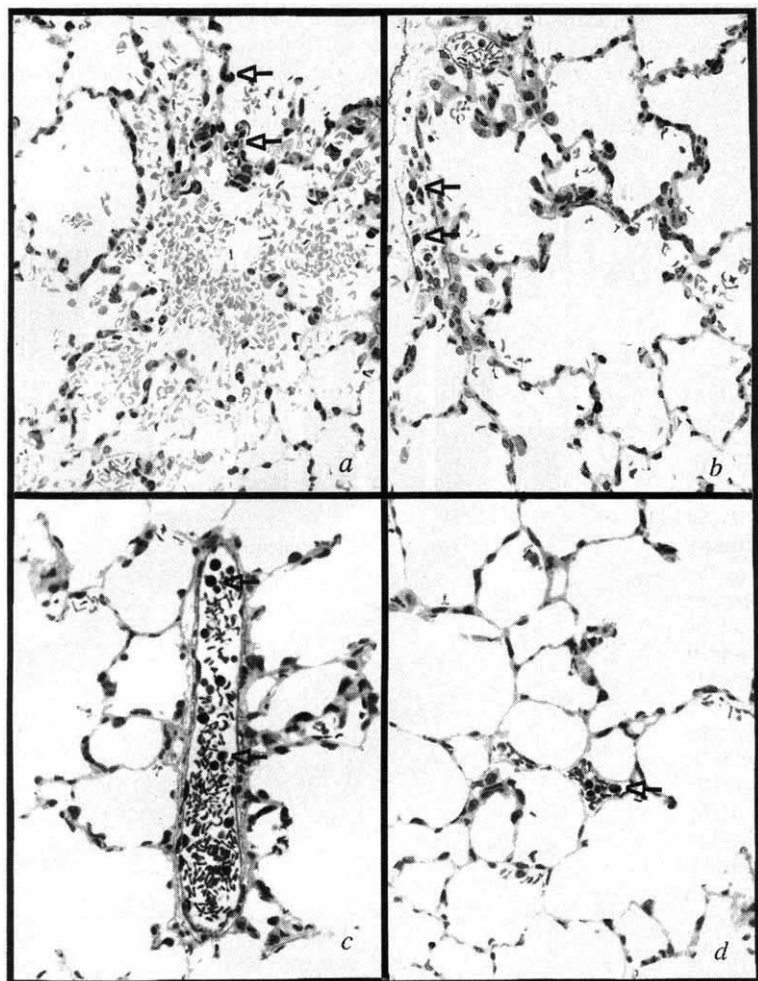


FIG. 4 Light micrographs of lungs from rats infused 30 min earlier with CVF and treated (5 min before infusion of CVF) with 200 μ g SLN-penta (a, b) or with 200 μ g SLX-penta (c, d), as described in Fig. 3. In a and b, there is extensive intra-alveolar haemorrhage; neutrophils (arrows) are close to the endothelial surfaces of interstitial capillaries (a) and venules (b). In animals treated with SLX-penta, there is little alveolar haemorrhage (c, d), and the intravascular neutrophils (arrows) are not close to the endothelium of venules (c) or interstitial capillaries (d). Preparation of morphological specimens is described elsewhere⁵. (Toluidine blue, all magnifications $\times 120$.)

Because there is evidence that SLX is a ligand for both E- and P-selectin^{8–11}, this raises the question as to whether or not it can be concluded that the protective effects of SLX in the CVF model of lung injury are exclusively P-selectin related. In CVF-infused rats pretreated with either E- or P-selectin-IgG chimaera, protection against injury occurred only in the case of P-selectin-IgG chimaera (M.S.M., S. R. Watson, C. Fennie and P.A.W., manuscript in preparation). These data support the concept that in the CVF model of lung injury the protective effects of SLX are related to its interaction with P-selectin and not with E-selectin.

In humans, the counterpart to the CVF model of acute lung injury may be adult respiratory distress syndrome (ARDS) in which neutrophils are present in alveoli, accompanied by evidence of oxidative inactivation of protein sulphhydryls¹². It is likely that ARDS is accompanied by complement activation, resulting in generation of C3a and C5a anaphylatoxins, leading to subsequent release of histamine, which upregulates endothelial P-selectin³. If the CVF model of lung injury is truly related to events in human ARDS, the SLX oligosaccharides may have therapeutic potential in the treatment of ARDS. □

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- Butcher, D. C. *Cell* **67**, 1033–1036 (1991).
- Lawrence, M. B. & Springer, T. A. *Cell* **65**, 859–873 (1991).
- Lorant, D. E. *et al.* *J. Cell Biol.* **115**, 223–234 (1991).
- Till, G. O., Johnson, K. J., Kunkel, R. & Ward, P. A. *J. clin. Invest.* **69**, 1126–1135 (1982).
- Mulligan, M. S. *et al.* *J. clin. Invest.* **90**, 1600–1607 (1992).
- Larsen, E. *et al.* *Cell* **59**, 305–312 (1989).
- Geng, J.-G. *et al.* *Nature* **343**, 757–760 (1990).
- Polley, M. J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **88**, 6224–6228 (1991).
- Zhou, Q. *et al.* *J. Cell Biol.* **115**, 557–564 (1991).
- Springer, T. A. & Lasky, L. A. *Nature* **349**, 196–197 (1991).
- Paulson, J. C. in *Adhesion: Its Role in Inflammatory Disease* 19–42 (Freeman, New York, 1992).

- Spragg, R. G. & Smith, R. M. in *The Lung: Scientific Foundation* (eds Crystal, R. G. & West, J. B.) 2003–2017 (Raven, New York, 1991).
- Ichikawa, Y. *et al.* *J. am. chem. Soc.* (in the press).
- Ball, G. E. *et al.* *J. am. chem. Soc.* **114**, 5449–5451 (1992).

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Bacterial and retroviral superantigens share a common binding region on class II MHC antigens

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STAPHYLOCOCCAL enterotoxin A (SEA), one of the most potent T-cell mitogens known, has been classified as a bacterial superantigen on the basis of ability to stimulate $V\beta$ -specific T-cell subsets¹. SEA interacts with class II major histocompatibility complex (MHC) antigens on antigen-presenting cells and the T-cell antigen receptor (TCR) on T cells²⁻⁴, resulting in a ternary complex of MHC-SEA-TCR. MIs antigens are known to be products of mouse mammary tumour virus (MMTV)^{5,6}, and it has been reported that two exogenous strains of MMTV encode retroviral superantigens in the open reading frames of the 3' long terminal repeat of the viral genome^{7,8}; however, no binding of the putative MMTV superantigen to either MHC antigens or TCR has been demonstrated. Here we use synthetic peptides to identify a site on the MMTV-1 superantigen that binds to class II MHC antigens. The site is encompassed by amino-acid residues 76-119 of the MMTV-1 superantigen. Direct binding and competition experiments show that the MMTV superantigen and SEA bind to at least one common region on class II MHC antigens.

Overlapping peptides (referred to as ORF (open reading frame) peptides) corresponding to the predicted extracellular domain of the MMTV-1 ORF superantigen⁸ were synthesized (Table 1). The ORF peptides were initially tested at a concentration of 200 μ M for their relative abilities to compete with ¹²⁵I-SEA for binding to A20 cells, which express I-A^d and I-E^d (ref. 9). ORF (76-119) reduced ¹²⁵I-SEA binding to A20 cells by 63%, whereas the other peptides had no effect (Fig. 1a). Thus, only one of the overlapping ORF peptides, ORF(76-119), significantly blocked ¹²⁵I-SEA binding to A20 cells.

Dose-response was studied on A20 cells with several ORF peptides, including ORF(76-119). ORF(76-119) reduced ¹²⁵I-SEA binding by 50% at a concentration of 20 μ M (Fig. 1b). Further, ORF(76-119) consistently competed with ¹²⁵I-SEA in a manner similar to unlabelled SEA, although SEA was 20 times more effective. Unlabelled SEA at a concentration of 1 μ M reduced ¹²⁵I-SEA binding to A20 cells by 50%. These data are consistent with the reported value of K_d for SEA binding to I-E^d of $\sim 10^{-6}$ M (ref. 10). A peptide corresponding to a C-terminal region of the MMTV superantigen, ORF(245-276), was not consistently a competitor for SEA binding. The peptide corresponding to the C-terminal tail, ORF(274-313), did not compete. A peptide having the same amino-acid content as ORF(76-119), but in a scrambled sequence (Table 1), did not compete (data not shown), indicating that ORF(76-119) competition was sequence-specific. No direct binding of ¹²⁵I-ORF(76-119) to SEA or related superantigens was detected (data not shown). Thus, ORF(76-119) binds specifically to A20 cells, and competes for SEA binding in a dose-dependent manner.

In direct binding studies, ¹²⁵I-ORF(76-119) bound to A20 cells and was effectively inhibited by both unlabelled SEA and ORF(76-119) (Fig. 1c). Unlabelled SEA and ORF(76-119) competed with ¹²⁵I-ORF(76-119) in a similar manner, although SEA was a more potent competitor. SEA reduced ¹²⁵I-ORF(76-

119) binding by 50% at 1.8 μ M, as compared to 25 μ M for unlabelled ORF(76-119). Neither the ORF(76-119) scrambled peptide nor the C-terminal ORF peptide competed, showing that ORF(76-119) binding was both sequence- and region-specific. Toxic shock syndrome toxin-1 (TSST-1) did not compete with ¹²⁵I-ORF(76-119), whereas the staphylococcal

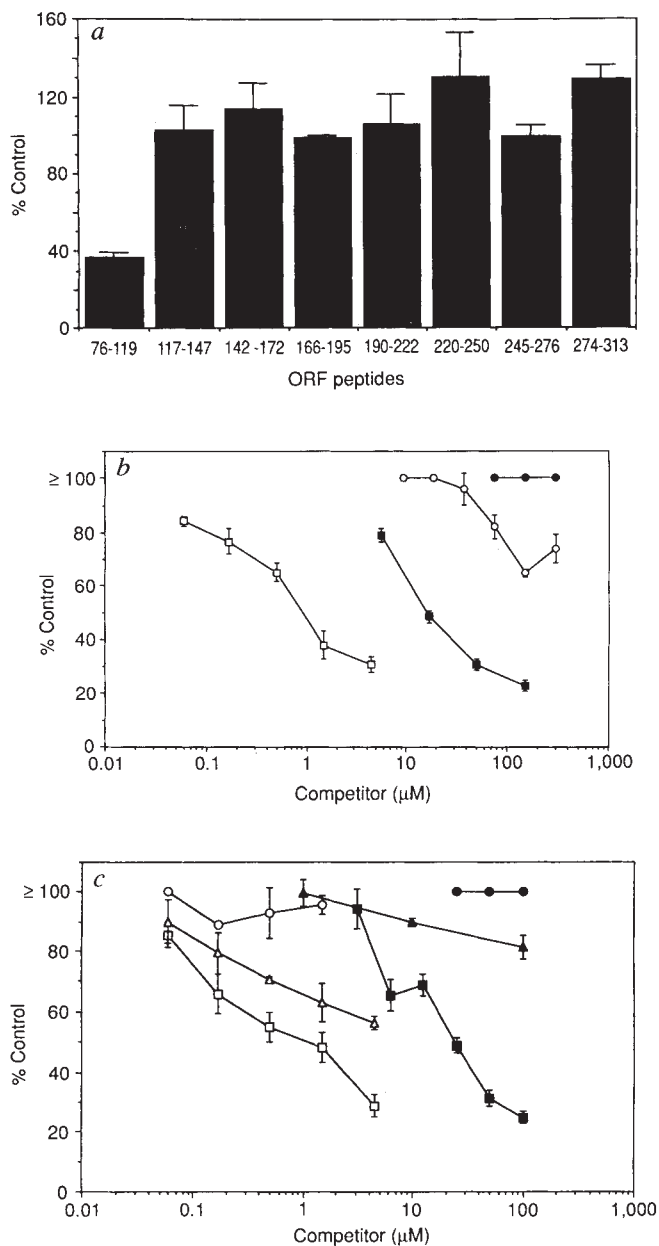


FIG. 1 a, Ability of ORF peptides to compete with ¹²⁵I-SEA for binding to A20 cells. SEA (Toxin Technology) was labelled with Na¹²⁵I (specific activity ~ 130 – 150 μ Ci per μ g) and binding was studied as described¹⁹. Binding of ¹²⁵I-SEA in the absence of competitors was $6,384 \pm 400$ c.p.m. Data represent the mean of three individual experiments, each performed in duplicate. Each bar represents the mean per cent reduction of SEA control binding in the presence of ORF peptides $\pm$ s.d. b, Ability of ORF(76-119) to compete with ¹²⁵I-SEA for binding to A20 cells in dose-response studies. ¹²⁵I-SEA was used at 2.5 nM. $\square$, SEA; $\blacksquare$, ORF(76-119); $\circ$, ORF(245-276); and $\bullet$, ORF(274-313). c, Ability of SEA, SEB, and TSST-1 to compete with ¹²⁵I-ORF(76-119) for binding to A20 cells. ¹²⁵I-ORF(76-119) (~ 30 – 60 μ Ci per μ g) was used at a final concentration of 2.3 nM. Binding of ¹²⁵I-ORF(76-119) in the absence of competitors was $4,556 \pm 448$ c.p.m. $\square$, SEA; $\blacksquare$, ORF(76-119); $\circ$, TSST-1; $\triangle$, SEB; $\blacktriangle$, ORF(76-119) scrambled peptide; and $\bullet$, ORF(274-313).

TABLE 1 Amino-acid sequences of ORF peptides

ORF peptide	Sequence
ORF(76–119)	DSFNSSVQDYNLNDSNSTFLLGQGQPQTSSYPKPHRLCPSEIE
ORF(117–147)	EIEIRMLAKNYIFTNETNPIGRLLIMMLRNE
ORF(142–172)	MMLRNESLSFSTIFTQIRLEMGIEKRKRRS
ORF(166–195)	ENRKRRTSVVEQVQGLRASGLEVKRGKRS
ORF(190–222)	KRGKRSALVKIGDRWWQPGTYRGPIYRPTDAP
ORF(220–250)	DAPLPYTGGRYDLNDFRWVTVNGYKVLRSPL
ORF(245–276)	LYRSLPFRERLARARPPWCVLSSQEEKDDMKQQ
ORF(274–313)	KQQVHDYIYLTGMHVKVFNYSREAKRHIEHIKALP
ORF(76–119) scrambled	PNSNEGLSQQSTDPSPHNFILSNENSYPCYSLLGDVQREDSTKF

Amino-acid sequence of overlapping ORF peptides. MMTV-1 ORF sequence was derived from ref. 7. A composite surface profile¹⁸ was generated; peptides were synthesized as described¹⁹. The ORF(76–119) scrambled peptide sequence was generated using the sequence edit program²⁰.

enterotoxin B (SEB) competed less effectively than SEA, which is consistent with their relative competitive abilities with SEA^{11,12}. Our results thus indicate that binding of ORF(76–119) to murine class II MHC antigens occurs at a region(s) to which SEA also binds.

Two further pieces of evidence indicate that ORF(76–119) binds to class II MHC antigens. Binding of ORF(76–119) to class II-negative mouse L cells was insignificant compared with mouse A20 cells, even at concentrations as high as 5 nM (Fig. 2, top). Monoclonal antibodies raised against class I MHC antigens had no effect on ¹²⁵I-ORF(76–119) binding to A20 cells (Fig. 2, bottom). Polyclonal anti-I-A^d and anti-I-E^d significantly blocked binding and in combination reduced binding to A20 cells by 73%. These data indicate that ORF(76–119), like

SEA¹⁰, binds to both I-A and I-E. An I-A^d β 1 helix-specific monoclonal antibody reduced binding by 30%, suggesting that this is a region on I-A to which ORF(76–119) binds. Polyclonal antibodies to I-A^k had minor effects on ORF(76–119) binding. These data thus indicate that ORF(76–119) binds to class II MHC antigens.

To determine directly whether ORF(76–119) binds to the β 1 helix of I-A, a competitive radioimmunoassay was done in which ¹²⁵I-SEA and ¹²⁵I-ORF(76–119) were tested for their relative abilities to bind to I-A β ^b(60–90) peptide. SEA and ORF(76–119), but not scrambled ORF(76–119) (data not shown), competed with both ¹²⁵I-SEA and ¹²⁵I-ORF(76–119)

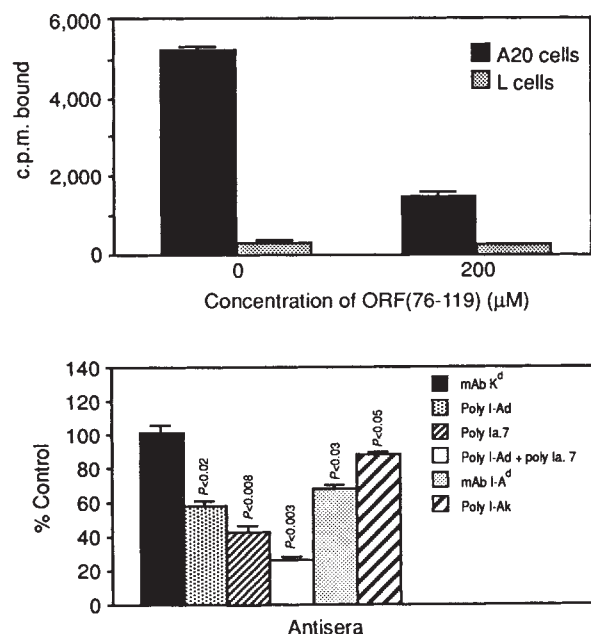


FIG. 2 Binding of ¹²⁵I-ORF(76–119) to class II-positive A20 cells and class II-negative L cells (top) and blockage of ¹²⁵I-ORF(76–119) binding to A20 cells by antibodies to class I and class II MHC antigens (bottom). ¹²⁵I-ORF(76–119) was used at a final concentration of 5 nM. Monoclonal antibodies (mAb) used in this study had similar potencies, as determined by Hybridoma Core Facility, Department of Pathology, University of Florida, and were used at a final dilution of 1/30. mAb K^d (34-1-2S; ref. 21) is specific for the m31 determinant of K^d; mAb I-A^d (MK-D6; ref. 10) is specific for the β 1 helix of I-A^d. Polyclonal antibodies were used at a final dilution of 1:100. Poly I-A^d (cat.Y1-9-04-26-01) is specific for the m11 determinant of I-A^d and poly Ia.7 (cat.Y1-7-02-02-01) is specific for the m7 determinant of I-E^d. Both antibodies were from NIAID Repository, Rockville, MD; poly I-A^k was from Accurate Chemicals, Westbury, NY.

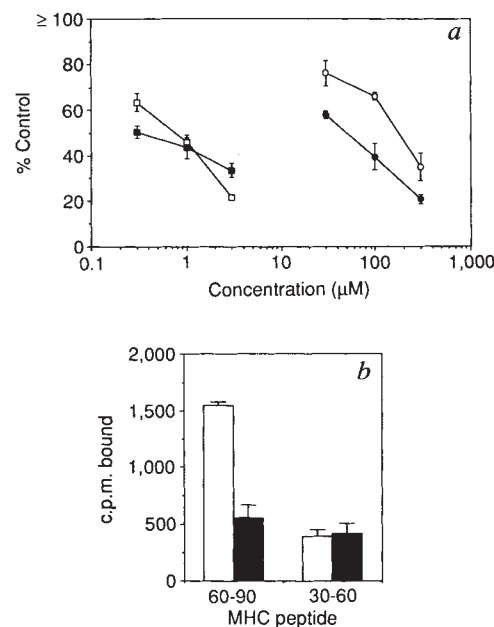


FIG. 3 Binding of ¹²⁵I-SEA and ¹²⁵I-ORF(76–119) to I-A β ^b(60–90) and inhibition of binding by ORF(76–119) (a) and relative ability of ORF(76–119) to bind I-A β ^b(60–90) and I-A β ^b(30–60) (b). The experiment was performed as previously described²², using replicates of six. a, Binding to I-A β ^b(60–90): ■, unlabelled SEA versus ¹²⁵I-SEA; ●, unlabelled ORF(76–119) versus ¹²⁵I-SEA; □, unlabelled SEA versus ¹²⁵I-ORF(76–119); ○, unlabelled ORF(76–119) versus ¹²⁵I-ORF(76–119). Each point represents the mean per cent reduction of SEA control binding in the presence of competitors $\pm$ s.d. Binding of ¹²⁵I-SEA and ¹²⁵I-ORF(76–119) in the absence of competitor was, 2,656 $\pm$ 92 c.p.m. and 1,547 $\pm$ 33 c.p.m., respectively. b, Binding of ¹²⁵I-ORF(76–119) to I-A β ^b(60–90) and I-A β ^b(30–60) in the presence (■) and absence (□) of excess unlabelled ORF(76–119). ¹²⁵I-ORF(76–119) and unlabelled ORF(76–119) were used at final concentrations of 5 nM and 300 μ M, respectively.

for binding to I-A β ^b(60–90) in a manner similar to the competition seen on whole cells (Fig. 3a). As previously shown for SEA^{13,14}, ORF(76–119) did not directly bind to I-A β ^b(30–60) (Fig. 3b). Thus SEA and ORF(76–119) bind to a similar region on the β -chain of the class II MHC molecule.

Viral superantigens may be involved in the infection processes of MMTV and human immunodeficiency virus (HIV)^{15,16}. Our results offer direct evidence that MMTV ORF protein can bind to MHC antigens, suggesting that bacterial and retroviral superantigens may act similarly.

MMTV superantigen is probably a 45K type II integral membrane protein with a glycosylated extracellular C terminus and an intracellular N terminus⁸. Although no direct evidence exists, the variability of the C-terminal residues of ORF proteins of various MMTV strains seems to correlate with the differences in V β specificity, suggesting that this region binds TCR⁷. There is a hydrophobic sequence near the N terminus (residues 45–64) which may act as a transmembrane region. But recent studies suggest that MMTV7 superantigen is synthesized as a precursor molecule, is proteolytically cleaved at an internal site (~ residue 164), and is expressed as an 18.5K surface protein consisting of the C-terminal residues¹⁷. Membrane association may involve 'tethering' to class II MHC antigens or to the N-terminal portion of the precursor molecule. These data point to the importance of direct binding studies, such as those presented here, in the determination of the structural basis for interaction of MMTV

superantigen with its MHC and TCR receptors. □

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1. Johnson, H.M., Russell, J.K. & Pontzer, C.H. *FASEB J.* **5**, 2706–2712 (1991).
2. Fischer, H., Dohlisten, M., Lindvall, M., Sjogren, H.-O. & Carlsson, R. *J. Immun.* **142**, 3151–3157 (1989).
3. Gascoigne, N.R.J. & Ames, K.T. *Proc. natn. Acad. Sci. U.S.A.* **88**, 613–616 (1991).
4. Pontzer, C.H., Irwin, M.J., Gascoigne, N.R.J. & Johnson, H.M. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7727–7731 (1992).
5. Choi, Y., Kappler, J.W. & Marrack, P. *Nature* **350**, 203–207 (1991).
6. Acha-Orbea, H. *et al.* *Nature* **350**, 207–211 (1991).
7. Pullen, A.M., Choi, Y., Kushnir, E., Kappler, J.W. & Marrack, P. *J. exp. Med.* **175**, 41–47 (1992).
8. Choi, Y., Marrack, P. & Kappler, J.W. *J. exp. Med.* **175**, 847–851 (1992).
9. Sette, A., Buus, S., Colon, S., Miles, C. & Grey, H.M. *J. Immun.* **142**, 35–40 (1989).
10. Lee, J.M. & Watts, T.H. *J. Immun.* **145**, 3360–3366 (1990).
11. Pontzer, C.H., Russell, J.K. & Johnson, H.M. *Proc. natn. Acad. Sci. U.S.A.* **88**, 125–128 (1991).
12. Fraser, J.D. *Nature* **339**, 221–223 (1989).
13. Russell, J.K., Pontzer, C.H. & Johnson, H.M. *Biochem. biophys. Res. Commun.* **168**, 696–701 (1990).
14. Russell, J.K., Pontzer, C.H. & Johnson, H.M. *Proc. natn. Acad. Sci. U.S.A.* **88**, 7228–7232 (1991).
15. Golovkina, T.V., Chervonsky, A., Dudley, J.P. & Ross, S.R. *Cell* **69**, 637–645 (1992).
16. Laurence, J.L., Hodstev, A.S. & Posnett, D.N. *Nature* **358**, 255–259 (1992).
17. Winslow, G.M., Scherer, M.T., Kappler, J.W. & Marrack, P. *Cell* **71**, 719–730 (1992).
18. Parker, J.M.R., Guo, D. & Hodges, R.S. *Biochemistry* **25**, 5425–5432 (1986).
19. Griggs, N.D., Pontzer, C.H., Jarpe, M.A. & Johnson, H.M. *J. Immun.* **148**, 2516–2521 (1991).
20. Devereaux, J., Haeblerli, P. & Smithies, O. *Nucleic Acids Res.* **12**, 387–395 (1984).
21. Ozato, K., Mayer, N.M. & Sachs, D.H. *Transplantation* **34**, 113–118 (1982).
22. Magazine, H.I. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 1237–1241 (1988).

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Endogenous superantigen expression controlled by a novel promoter in the MMTV long terminal repeat

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ENDOGENOUS superantigens are encoded by the open reading frame contained within the mouse mammary tumour virus long terminal repeat (MMTV LTR). Superantigen expression results in T-cell proliferation and, during early ontogeny, T-cell deletion^{1,2}. Here we identify a novel promoter located upstream of the previously described MMTV promoter. Transcripts from this promoter initiate within the U3 region of the MMTV LTR and splice to the acceptor for endogenous superantigen coding region. The novel U3 promoter is active in B lymphocytes, which are cognate antigen-presenting cells for endogenous superantigen, and is able to direct expression of superantigen in the absence of the previously described MMTV promoter.

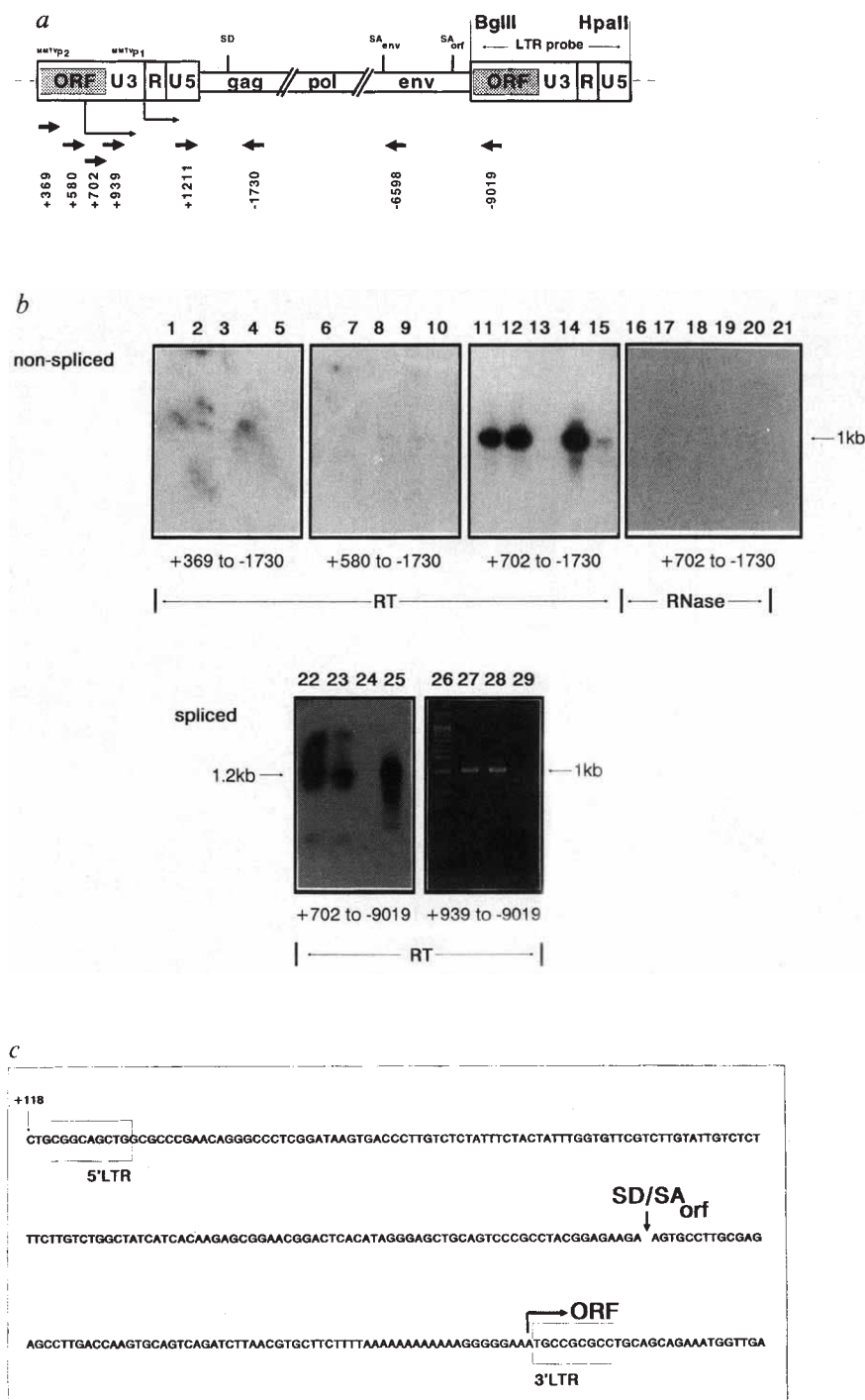
The long U3 region of the MMTV LTR contains an open reading frame (ORF)^{3,4}, regulatory sequences involved in hormone-responsive gene transcription^{5–7} and various positive and negative regulatory elements⁸. As well as encoding endogenous superantigen (Sag)^{1,2}, ORF encodes a negative-acting factor (Naf) which regulates viral transcriptional activity^{8,9}. While characterizing messenger RNAs encoded by *sag* and *naf*, we observed transcripts that included sequences from the U3 region of the 5' LTR, that is, 5' of the previously described transcription initiation site which is located at the U3-R boundary⁵ (Fig. 1a).

To investigate this, complementary DNA was prepared from cell lines either infected or transfected with MMTV and used for polymerase chain reaction (PCR) analysis. MMTV-specific, reverse-transcriptase-dependent PCR products could be detected using one primer located within the U3 region of the LTR (+702) and a second (–1,730) located in the *gag* gene (Fig. 1a and b). Direct sequencing of this PCR product identified it as identical to the known MMTV sequence between the two primers used in the PCR analysis. These results suggest that a transcript initiates within the U3 region of the MMTV LTR. In contrast, primers located further upstream (+369 and +580), used in combination with the primer located within the *gag* gene (–1,730), failed to give MMTV-specific PCR products (Fig. 1a and b). This suggested that a novel site of transcription initiation is located between +580 and +702.

The exact position of this putative initiation site was determined using RNA isolated from the MMTV-producing cell line GR (Fig. 2) and from MMTV transfected cell lines (not shown) in an S1 protection analysis. An end-labelled MMTV *Bst*YI DNA fragment covering this region (Fig. 2a) protected an MMTV-specific, U3 fragment of 45 nucleotides (nt) (Fig. 2b). This places the novel start site 45 nt 5' of the *Bst*YI site, that is, –498 relative to the previously described start (Fig. 1a). Additional minor start sites can be identified at positions between –502 and –493 (Fig. 2b). The location of the novel transcription initiation site was verified by primer extension analysis (Fig. 2c and d). The amount of RNA initiating at this site was enhanced when the cells were grown in the presence of the glucocorticoid hormone dexamethasone (Fig. 2b), presumably because of the hormone responsive element (HRE) located between –50 and –202, which is known to affect promoters in its vicinity^{5–7}. Candidate TATA and CAAT boxes can be found in a correct context upstream of the newly identified initiation site (Fig. 2c) as part of a putative novel MMTV promoter (^{MMTV}P2).

To determine whether transcripts from ^{MMTV}P2 use the described MMTV splice donor and acceptor sites (Fig. 1a; SD and SA), PCR analyses were done using the +702 primer and a primer (Fig. 1a; –9,019) located 3' of the splice acceptor site (SA_{orf}) described for the generation of ORF transcripts^{10,11} or a primer (Fig. 1a; –6,598) located 3' of the splice acceptor site (SA_{env}) described for the *env* mRNA^{12,13}. Unexpectedly, only

FIG. 1 The U3 region of the MMTV LTR contains a novel promoter. **a**, Schematic representation of an MMTV provirus. Two MMTV LTRs consisting of the U3 region containing the ORF; the R and U5 region flanks the viral structural genes *gag*, *pol* and *env*. The position of the MMTV promoter and associated transcription initiation site ($^{MMTV}P1$) are shown, as is the position of the novel promoter and initiation site ($^{MMTV}P2$) identified here. A series of primers (+369, +580, +702, +939, +1,211, -1,730, -6,598 and -9,019) used for PCR are shown. The numbers refer to the location of the primers relative to the 5' end of the MMTV provirus; + and - primers are complementary to the coding and anticoding strand, respectively. Also shown are the previously mapped splice donor (SD) and splice acceptors for the *env* (SA_{env}) and ORF (SA_{orf}) mRNAs. A *Bgl*II-*Hpa*II LTR-specific fragment that was used as a hybridization probe is indicated in the 3' LTR. **b**, Total cellular RNA was isolated from MMTV-producing GR cells²¹ (lanes 4, 9, 14, 19, 21 and 25), chronically MMTV-infected M1.19 cells²² (lanes 5, 10, 15 and 20), two independent CK²³ cell clones (CKmtv 1 and 2) transfected with an MMTV provirus (lanes 1, 2, 6, 7, 11, 12, 16, 17, 22, 23, 27, and 28), and from non-transfected CK cells (lanes 3, 8, 13, 18, 24 and 29). RNA was subjected to first-strand cDNA synthesis using an oligo(dT) primer (lanes 1 to 15, 22 to 25, and 27 to 29), to RNase treatment (lanes 16 to 20), or left untreated (lane 21); for PCR we used the primer pairs +369/-1,730 (lanes 1-5), +580/-1,730 (lanes 6-10), +702/-1,730 (lanes 11-15), +702/-9,019 (lanes 22 to 25) or +939/-9,019 (lanes 27 to 29). A 1-kb ladder marker (BRL) is shown in lane 26. The PCR products were analysed by gel electrophoresis, transferred to a zeta-probe filter and hybridized to the ³²P-labelled *Bgl*II-*Hpa*II (a) MMTV LTR-specific probe (lanes 1 to 25), or directly visualized by ethidium bromide staining (lanes 26 to 29). MMTV-specific PCR products corresponding to unspliced transcripts could be detected from MMTV-containing cells using primer pair +702/-1,730 (lanes 11, 12, 14 and 15), but not from non-MMTV-containing cells (lane 13) or using the other primer pairs (lanes 1 to 10). These PCR products could not be detected if the samples were pretreated with RNase (lanes 16 to 20) or if they were not subjected to cDNA synthesis (lane 21), indicating that they do not arise from proviral DNA. PCR products were identified by direct sequencing (not shown). PCR products corresponding to spliced transcripts could be detected from



MMTV-containing cells using the primer pairs +702/-9,019 (lanes 22, 23 and 25) and +939/-9,019 (lanes 27 and 28), but not from non-MMTV containing cells (lanes 24 and 29). **c**, The identity of the PCR products arising from transcripts directed by $^{MMTV}P2$ and using the described splice donor and acceptor for ORF (SD/SA_{orf}) was confirmed by direct sequencing. Part of this sequence, from the PCR product shown in lane 28, beginning at +118 relative to the previously described initiation site and consisting of the 3' end of the 5' LTR and the 5' end of the 3' LTR, is shown.

the ORF specific primer (-9,019) yielded a PCR product (Fig. 1b). A second primer (+939; Fig. 1a) also only yielded a PCR product (Fig. 1b) in conjunction with the ORF-specific primer (-9,019) and not with the *env* primer. Sequencing of these products revealed that the previously described SD and SA_{orf}

were used (Fig. 1a and c). These findings suggested to us that transcripts initiating from $^{MMTV}P2$ might specifically give rise to MMTV ORF-encoded Sag activity.

To investigate whether $^{MMTV}P2$ is active in spleen-derived cells, an S1 protection analysis was carried out using two

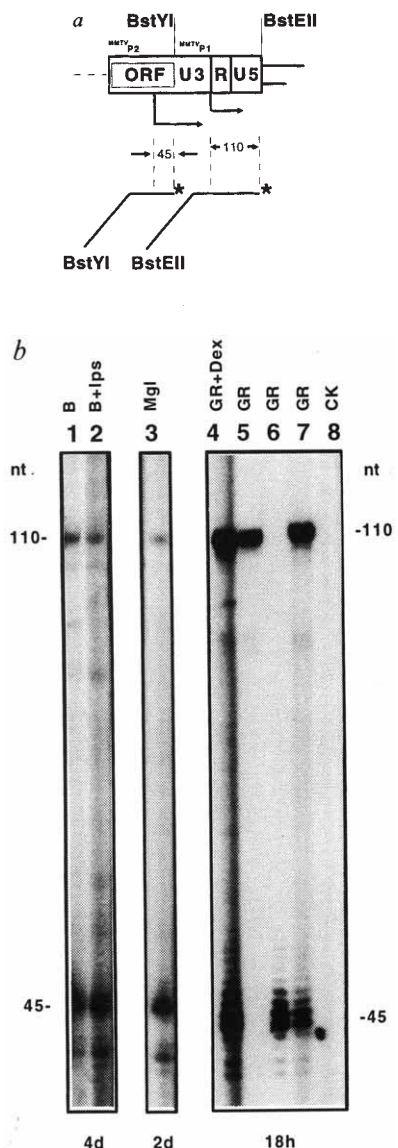
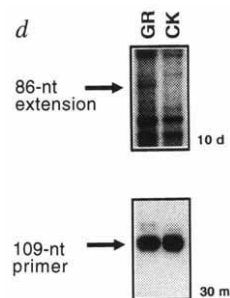
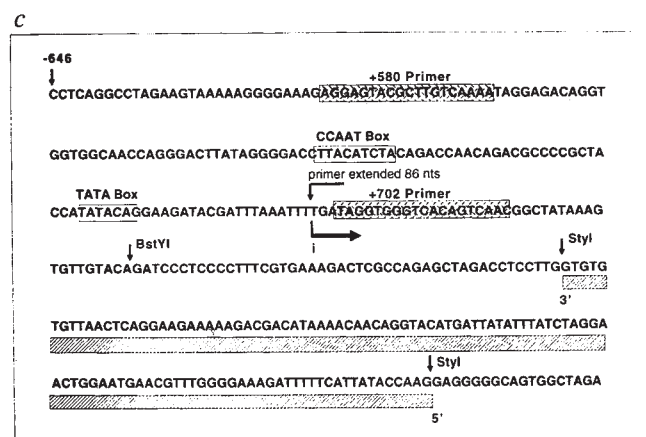


FIG. 2 Analysis of transcripts originating from the MMTV-LTR. *a*, Schematic representation of the MMTV LTR and the non-overlapping probes used for S1 protection analyses. A *BstEII* DNA fragment was used to map the initiation site of transcripts from the MMTV promoter ($^{MMTV}P1$). A *BstYI* DNA fragment was used to map transcripts from the novel U3 promoter ($^{MMTV}P2$). The sizes of the protected fragments (45 and 110 nt) from these probes are indicated, as is the position of the ^{32}P -labelled end (*). *b*, To determine the site of initiation of RNA from $^{MMTV}P2$, 20 μ g total RNA, isolated from GR cells (GR) grown in the absence (lanes 5–7) or presence (lane 4) of the synthetic glucocorticoid hormone dexamethasone (Dex), was hybridized to the end-labelled *BstYI* DNA fragment covering the implicated U3 region and/or the end-labelled *BstEII* fragment spanning the previously described MMTV initiation site. RNA–DNA adducts were digested with nuclease S1 and the double-stranded S1-resistant fragment was separated on an 8% denaturing polyacrylamide sequencing gel. When the *BstYI* probe alone is used (lane 6), a fragment of 45 nt can be detected which defines the novel transcription initiation site directed by $^{MMTV}P2$ at –498 relative to the MMTV initiation site (*c*). Additional bands around the major band at 45 nt are not artefacts and may represent a stuttering of the polymerase. Using the *BstEII* probe alone (lane 5), only the expected 110-nt fragment is visible. When both probes are used (lanes 4 and 7), both starts can be visualized. Treatment of the cells with dexamethasone (lane 4), results in enhanced transcription from both promoters. Neither the 110-nt signal, nor the 45-nt signal can be detected using RNA isolated from non-MMTV containing CK cells (lane 8). To determine whether the two MMTV promoters are active in B cells and mammary gland *in vivo*, 60 μ g RNA from resting



(lane 1) or LPS-stimulated (lane 2) B cells and 20 μ g RNA from non-virgin mammary gland (lane 3) were used in an S1 protection analysis with both probes as already described. The 110-nt fragment, indicative of transcription directed by $^{MMTV}P1$, as well as the 45-nt fragment representing transcripts from $^{MMTV}P2$, can be detected (lanes 1–3). Treatment of B cells with LPS results in a slight (2-fold) increase in transcription from $^{MMTV}P2$, but not from $^{MMTV}P1$ (lane 2). Below the lanes the exposure times are indicated of the X-ray film to the radioactive gel in days or hours. *c*, The sequence of the region containing the novel $^{MMTV}P2$ promoter and initiation site. The major site of initiation of transcription, mapped by the S1 protection analysis, is indicated by an arrow and 'i'. The sequences of the +580 and +702 primers, as well as potential TATA and CCAAT sequences are boxed. The numbers of base pairs indicated are relative to the position of the previously described transcription initiation site. The *BstYI* site used in the S1 protection analysis is indicated, as is the 109-bp *StyI* restriction fragment (shaded box) used for primer extension analysis. The location of the 86-nt primer extension is shown and corresponds to the initiation site as determined by S1 mapping. *d*, To confirm the location of the novel transcription initiation site, 50 μ g total RNA isolated from GR and CK cells was hybridized to the 109-bp ^{32}P -end-labelled *StyI* fragment and extended using reverse transcriptase. The products of the primer extension were analysed on a 6% denaturing polyacrylamide sequencing gel. The extended product of 195-nt, corresponding to an extension of 86-nt, can be detected in GR and MMTV-transfected CK cells, but not in non-transfected CK cells; this is indicated, as is the *StyI* restriction fragment primer, to show equal loading of the gel. Exposure times of the autoradiographs are indicated in days or min. Minor products can be detected around the 86-nt extended fragment, corroborating the stuttering observed in the S1 analysis.

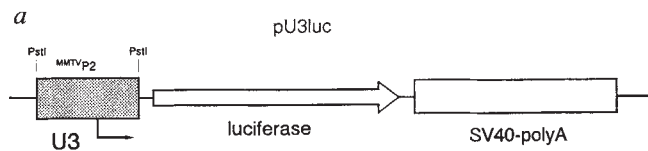
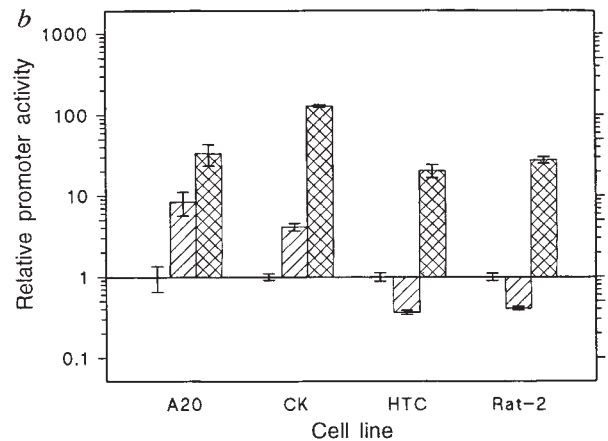


FIG. 3 The novel U3 promoter is functional in cells of the B lineage. *a*, The plasmid pU3luc was constructed by ligating a *Pst*I fragment containing part of the U3 region of the MMTV LTR carrying the novel promoter, to a promoterless luciferase gene linked to the termination signals from SV40 (pLUC1). *b*, A20 murine B-lymphoma-derived cells, rat hepatoma (HTC), fibroblast (Rat-2), and feline kidney (CK) cells were transiently transfected with 10 μ g of the following plasmids: pT109luc, carrying a luciferase gene under the transcriptional control sequences of the herpes simplex virus thymidine kinase (HSV-tk) promoter²⁴, pLUC1, carrying a 'promoterless' luciferase gene, or pU3luc described above. Cell extracts were prepared 48 h post-transfection and equivalent amounts used for luciferase assay as described²⁵ using a Berthold AutoLumat LB 953. The luciferase activities obtained relative to the 'promoterless' pLUC1 (background



activity set at 1) in each cell type reflect the relative promoter activity after transfection of pU3luc (diagonal stripes) and pT109luc (cross-hatched). The results reflect the mean and s.e.m. from 4 experiments and reveal that while the HSV-tk promoter in pT109luc is active in all 4 cell types, the novel MMTV promoter, ^{MMTV}P2 in pU3luc, shows greatest activity in A20 cells and moderate activity in CK cells.

non-overlapping probes simultaneously: the *Bst*YI fragment already described (Fig. 2a) and a *Bst*EII fragment (Fig. 2a) that protects a 110-nucleotide region corresponding to the previously described MMTV promoter (^{MMTV}P1) initiation site. Both of the promoters were used in non-virgin mammary gland as well as in both quiescent and lipopolysaccharide (LPS)-stimulated, spleen-derived blasts (Fig. 2b). Transcription from ^{MMTV}P2 in blasts was slightly enhanced (2-fold) by treatment with LPS, an agent known to upregulate lymphocyte specific promoters. In contrast, ^{MMTV}P1 was not influenced by LPS treatment.

The ability of ^{MMTV}P2 to direct the expression of a heterologous gene *in vitro* was also investigated. A fragment of the U3 region of the MMTV LTR containing ^{MMTV}P2, but lacking ^{MMTV}P1, was cloned in front of a promoterless luciferase gene. The resulting construct, pU3luc, in which luciferase expression is driven by ^{MMTV}P2 (Fig. 3a), a promoterless luciferase-containing plasmid, pLUC1, and a plasmid carrying the luciferase gene transcribed from a thymidine kinase promoter, pT109luc, were individually transfected into various cell types. ^{MMTV}P2 directed relatively high levels of expression of luciferase in a B-lymphocyte-derived cell line, A20, and moderate levels of expression in CK cells (Fig. 3b), one of the few cell lines permissive for MMTV¹⁴. No promoter activity could be detected

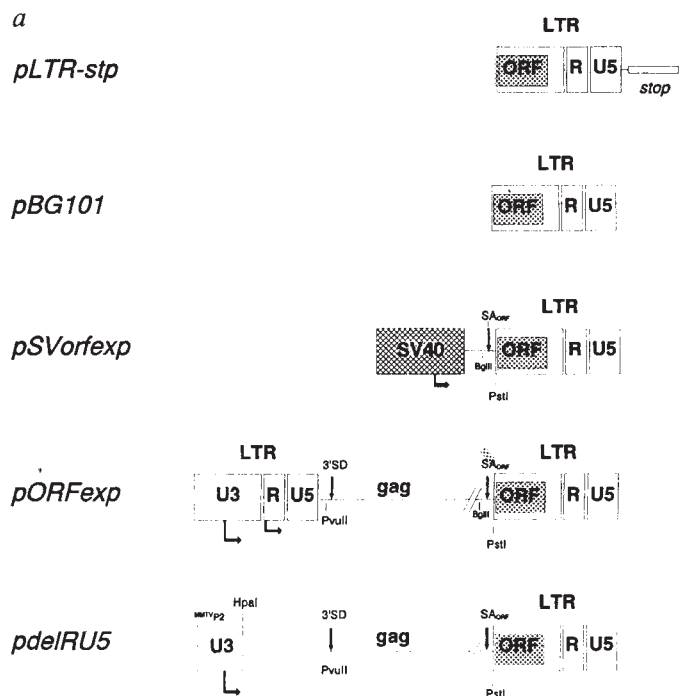
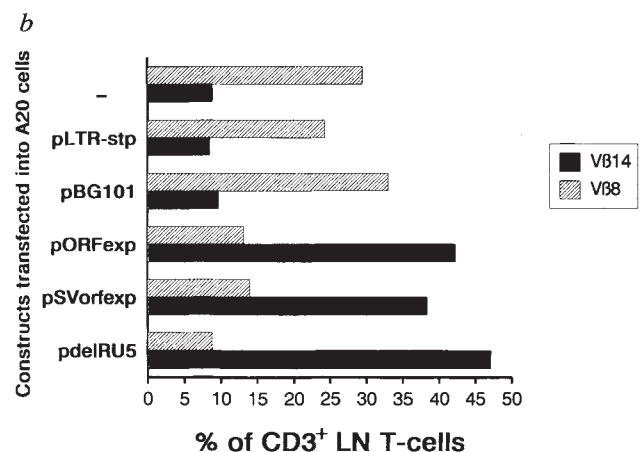


FIG. 4 The novel U3 promoter can direct efficient superantigen expression. *a*, Constructs used for superantigen assays. Two promoterless Mtv-2 LTR-containing constructs, pLTR-stp with additional polyadenylation signals (stop), and pBG101 without these signals²⁶, were used to demonstrate the inactivity of the MMTV LTR in the absence of additional promoter sequences. The pSVorfexp construct consists of an SV40 promoter coupled to a complete Mtv-2 LTR. pORFexp consists of an MMTV 5'LTR containing ^{MMTV}P1 and ^{MMTV}P2, part of the *gag* coding sequences and the 3'LTR of Mtv-2. The construct pdelRU5 is derived from pORFexp and lacks the ^{MMTV}P1, R and U5 regions of the 5'LTR. *b*, A20 cells were transiently transfected with 20 μ g of the constructs indicated and 20 h later were irradiated (3,000 rad); 1×10^7 transfected A20 cells were co-cultured with 2×10^6 primary T cells freshly isolated from popliteal lymph nodes (LN) from BALB/c mice. After 4 d of culture surviving T cells were stained with R-phycoerythrin-labelled anti-CD3 mAb and either FITC-conjugated anti-V β 8 mAb or anti-V β 14 mAb, and analysed by FACS (Elite, Coulter Inc.). The percentage of CD3⁺ T-cells that are V β 8⁺ or V β 14⁺ are shown. A decrease in the percentage of V β 8⁺ cells indicates the outgrowth of another V β -bearing class of T cells. An increase in the percentage of V β 14⁺ cells demonstrates that the transfected A20 cells express the Mtv-2 superantigen.



from the pU3luc construct upon transfection into HTC or Rat-2 cells.

The usage of ^{MMTV}P2 in B-cells suggested that this promoter may be driving the expression of the MMTV-encoded Sag. To determine this, an MMTV-containing plasmid that showed superantigen expression was modified by deletion of ^{MMTV}P1, leaving only ^{MMTV}P2 to drive transcription (pdelRU5). This, and control plasmids, were transiently transfected into A20 cells and the cells assayed for superantigen activity. The Sag encoding 3' LTR in all constructs was derived from the Mtv-2 locus, which has been shown to stimulate V β 14 bearing T cells² (Fig. 4a). Introduction of the ^{MMTV}P2 driven construct caused a selective proliferation of ligand-reactive V β 14 T-cells, and their clonal expansion from 8 to 46% in a 4-day culture (Fig. 4b). We conclude that the ^{MMTV}P2 promoter is able to drive Sag expression.

The data presented in this paper demonstrates that a novel U3 promoter (^{MMTV}P2) directs Sag expression in B cells. First, transcripts originating from this promoter splice directly to the previously described acceptor site for ORF^{10,11}. Second, this promoter is active in B-lymphocyte-derived cells *in vitro* and in LPS-stimulated blasts *ex vivo*, which function as antigen-presenting cells for Sag-mediated effects. Finally, this promoter is able to direct efficient Sag activity in the absence of the previously described MMTV promoter. The identification of a second promoter is a major finding as, to our knowledge, this is the first time an additional promoter has been described in the LTR of any retrovirus. The life cycle of MMTV requires that milk-borne virus be transported from the gut to the mammary gland. Evidence has been presented that both T and B cells are essential for this^{8,15-20}. We speculate that ^{MMTV}P2 may be the

major promoter used in B cells for expression of Sag, which leads to a concomitant amplification of B and T cells, thereby increasing the virus reservoir and its chance to reach the mammary gland. □

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- Choi, Y., Kappler, J. W. & Marrack, P. *Nature* **350**, 203-207 (1991).
- Acha-Orbea, H. et al. *Nature* **350**, 207-211 (1991).
- Donehower, L. A., Huang, A. L. & Hager, G. L. *J. Virol.* **37**, 226-238 (1981).
- Kennedy, N. et al. *Nature* **295**, 622-624 (1982).
- Hynes, N. E. et al. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3637-3641 (1983).
- Buetti, E. & Diggelmann, H. *EMBO J.* **2**, 1423-1429 (1983).
- Majors, J. E. & Varmus, H. E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5866-5870 (1983).
- Günzburg, W. H. & Salmons, B. *Biochem. J.* **283**, 625-632 (1992).
- Salmons, B., Erfle, V., Brem, G. & Günzburg, W. H. *J. Virol.* **64**, 6355-6359 (1990).
- Wheeler, D. A., Butel, J. S., Medina, D., Cardiff, R. D. & Hager, G. L. *J. Virol.* **46**, 42-49 (1983).
- van Ooyen, A. J., Michalides, R. J. & Nusse, R. *J. Virol.* **46**, 362-370 (1983).
- Redmond, S. M. S. & Dickson, C. *EMBO J.* **2**, 125-131 (1983).
- Majors, J. E. & Varmus, H. E. *J. Virol.* **47**, 495-504 (1983).
- Salmons, B., Groner, B., Calberg-Bacq, C. M. & Ponta, H. *Virology* **144**, 101-114 (1985).
- Liegler, T. J. & Blair, P. B. *J. Virol.* **59**, 159-162 (1986).
- Tsubura, A. et al. *Cancer Res.* **48**, 6555-6559 (1988).
- Acha-Orbea, H. & Palmer, E. *Immun. Today* **12**, 356-361 (1991).
- Lund, F. E. & Corley, R. B. *J. exp. med.* **174**, 1439-1450 (1992).
- Huber, B. T. *Curr. Biol.* **2**, 495-496 (1992).
- Held, W. et al. *J. exp. Med.* **177**, 359-366 (1993).
- Ringold, G. M., Yamamoto, K. R., Tomkins, G. M., Bishop, M. & Varmus, H. E. *Cell* **6**, 299-305 (1975).
- Ringold, G. M., Yamamoto, K. R., Shank, P. R. & Varmus, H. E. *Cell* **10**, 19-26 (1977).
- Crandell, R. A., Fabricant, C. G. & Nelson-Rees, W. A. *In vitro* **9**, 176-185 (1973).
- Nordeen, S. K. *Biotechniques* **6**, 454-458 (1988).
- Hornsby, P., Yang, L., Lala, D. S., Cheng, C. Y. & Salmons, B. *Biotechniques* **12**, 244-251 (1992).
- Jaggi, R., Salmons, B., Muellener, D. & Groner, B. *EMBO J.* **6**, 2609-2616 (1986).

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Transporter-independent processing of HIV-1 envelope protein for recognition by CD8⁺ T cells

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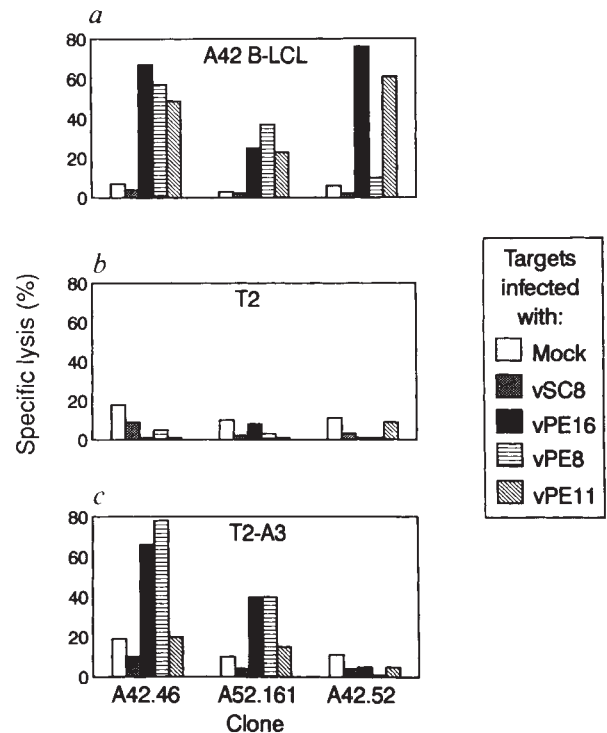
CD8⁺ cytolytic T lymphocytes (CTL) identify virally infected cells by recognizing processed viral antigen in association with class I major histocompatibility complex (MHC) molecules on infected cells¹⁻⁴. Processing begins in the cytosol⁵⁻⁷ with the generation of peptides, possibly by a protease complex with MHC-encoded subunits, known as the proteasome⁸⁻¹¹. Transport of the resulting cytosolic peptides into the endoplasmic reticulum for association with class I molecules is essential and probably involves a heterodimer of the MHC-encoded proteins, Tap-1 and Tap-2¹²⁻¹⁷. The site of processing of viral envelope proteins is uncertain. These proteins are not present in the cytosol because of cotranslational translocation into the endoplasmic reticulum. We show here that the HIV-1 envelope (env) protein is processed in infected cells by a novel Tap-1/Tap-2-independent pathway that seems to be localized to the endoplasmic reticulum.

CD8⁺ CTL specific for the HIV-1 env protein contribute to host defence by lysing infected cells presenting processed env peptides in association with class I MHC molecules¹⁸⁻²⁰. Env-specific CD8⁺ human CTL clones were isolated to study processing of the env protein. These clones showed antigen-specific cytolytic activity against histocompatible B lymphoblastoid cell lines

(B-LCL) infected with vaccinia vectors carrying wild-type and truncated forms of the HIV-1 env gene (Fig. 1a). The clones also lysed B-LCL expressing a cytosolic env protein lacking the amino-terminal signal sequence. Thus env peptides generated in the cytosol can be transported into the endoplasmic reticulum (ER) lumen for association with class I molecules. However, it remained unclear whether normal processing of wild-type env protein was dependent on the transport of cytosolic peptides into the ER, as is the case with almost all viral proteins studied to date. Thus we expressed env protein in T2 cells^{21,22}, which have a homozygous deletion of a portion of the MHC that includes genes for Tap-1 and Tap-2. T2 cells fail to present cytosolic viral antigens to CD8⁺ CTL²³⁻²⁵, and because assembly and surface expression of class I MHC molecules are peptide dependent²⁶, these cells have low levels of surface class I. T2 cells expressing various forms of the HIV-1 env gene were not lysed by human leukocyte antigen (HLA)A3.1- or B35-restricted clones because T2 cells lack the correct MHC restriction elements (Fig. 1b). Therefore, CTL assays were also done with T2 cells transfected with a genomic clone of HLA A3.1²⁷. These T2-A3 transfectants synthesize A3.1, but like endogenous B5 and several other human class I molecules expressed in T2 cells, it is not expressed on the cell surface^{21,23,24}. T2-A3 cells expressing wild-type env protein or secreted gp120 were readily lysed by two independent A3.1-restricted clones specific for epitopes in gp120 (Fig. 1c). When the cytosolic form of the env protein was expressed in T2-A3 cells, there was no significant lysis by either A3.1-restricted CTL clone. In addition, T2-A3 cells infected with a vaccinia vector carrying the HIV-1 nef gene were not lysed by an A3.1-restricted, nef-specific CTL clone. The same clone lysed T2-A3 cells pulsed with the relevant nef peptide and A3.1⁺ B-LCL infected with the nef vector (data not shown). These results confirm the inability of T2-A3 cells to process and present cytoplasmic viral proteins. The fact that T2-A3 cells can nevertheless present wild-type and secreted forms of the env protein demonstrates that processing of the env protein does not depend on

FIG. 1. Transporter-independent processing of the HIV-1 env protein. CD8⁺ CTL clones were tested for the lytic activity against histocompatible EBV-transformed B-LCL or transporter-defective T2 cells infected with vaccinia virus vectors carrying various forms of the HIV-1 env gene. A42.46 and A52.161 are HLA A3.1-restricted clones derived from two different donors (A42 and A52). A42.52 is an HLA B35-restricted clone from donor A42. vSC8 is a control vaccinia vector lacking HIV-1 genes. The vPE16 vector carries the wild-type HIV-1 env gene (LAI isolate)⁴³. vPE8 carries a truncated env gene with a stop codon at the 3' end of the coding sequence for the N-terminal gp120 subunit of the env protein⁴⁴. Cells infected with vPE8 secrete gp120^{44,45}. vPE11 carries a mutant form of the env gene in which codons for the N-terminal signal sequence have been deleted⁴⁵. In vPE11-infected cells, env protein is synthesized and rapidly degraded in the cytosol⁴⁵. **a**, Lysis of histocompatible A42 B-LCL (HLA class I genotype: A3, A11, B18, B35, Bw4, Bw6, Cw1, Cw5) expressing wild-type, secreted, and cytoplasmic forms of the env protein by CD8⁺ CTL clones. Note that only clones A42.46 and A52.161 lyse vPE8-infected targets indicating specificity for epitopes in the gp120; clone A42.52 is specific for an epitope in the C-terminal gp41 subunit. **b**, Env-specific A3.1- and B35-restricted CTL clones do not lyse env-expressing T2 cells (genotype A2.1, B5) which lack the correct restriction element. **c**, Env-specific A3.1-restricted clones lyse T2-A3 cells expressing wild-type and secreted env protein, but not T2-A3 cells expressing cytoplasmic env protein.

METHODS. CTL clones were isolated as described elsewhere⁴⁶. Class I restriction and epitope specificity were determined by standard methods as described in detail elsewhere (P. Johnson, S.A.H., R.F.S., and B. Walker, manuscript in preparation). Vaccinia vectors have been previously described^{43, 45}. Lysis was measured in a standard ⁵¹Cr release assay at an E:T ratio of 1:1. Target cells infected with ultraviolet-inactivated vaccinia vectors were not lysed, ruling out the possibility that the observed lysis was due to trace env peptides contaminating the vaccinia stocks (data not shown).



the Tap-1 and Tap-2 gene products and can occur entirely in the ER or a post-ER compartment. Moreover, the high levels of lysis of env-expressing T2-A3 cells indicate that the degree of processing by this novel pathway is sufficient to account for all of the processing that occurs in transporter-competent cells (Fig. 1c).

We also compared lysis of env-expressing T2-A3 cells with the lysis of T2-A3 cells pulsed with a high concentration of the appropriate epitopic peptide (Fig. 2). Exogenous epitopic peptide can overcome the defect in transporter-mutant cells and restore assembly and surface expression of MHC class I molecules²⁶. Lysis of env-expressing T2-A3 cells was equivalent to the maximal level of lysis achievable with exogenous epitopic peptide, confirming that the transporter-independent processing

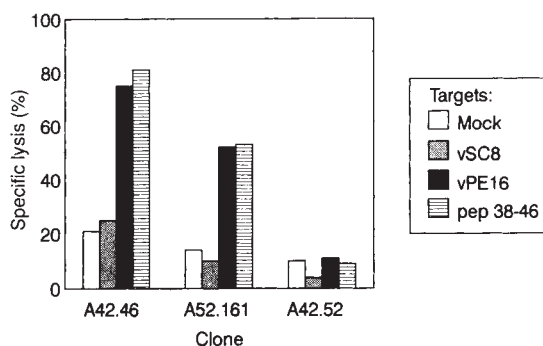


FIG. 2. Comparison of transporter-independent processing and exogenous peptide as sources of env epitopes recognized by HLA A3.1-restricted CTL clones. T2-A3 cells were mock-infected, infected with vSC8 or vPE16 as described in the legend to Fig. 1, or pulsed for 2 h with a synthetic A3.1-restricted epitopic env peptide at a concentration giving maximal lysis (100 μ g ml⁻¹). Lysis was measured in a standard ⁵¹Cr release assay at an E:T ratio of 1:1.

mechanism identified here represents a major antigen processing pathway for the env protein. To verify that the results obtained with vaccinia vectors provided an accurate model for processing reactions occurring in HIV-1-infected cells, CTL clones were tested for lytic activity against HIV-1-infected T2-A3 cells (Fig. 3). A high level of antigen-specific, MHC-dependent lysis of HIV-1-infected T2-A3 cells was detected.

The above results demonstrate that the HIV-1 env protein can be processed by a mechanism that does not depend on transport of peptides from the cytosol into the ER by Tap-1 and Tap-2. To determine whether processing of the env protein takes place in the ER or in a post-ER compartment, we examined the effect of retaining the env protein in the ER on presentation of env epitopes to CTL. Coexpression of an ER-retained form of CD4

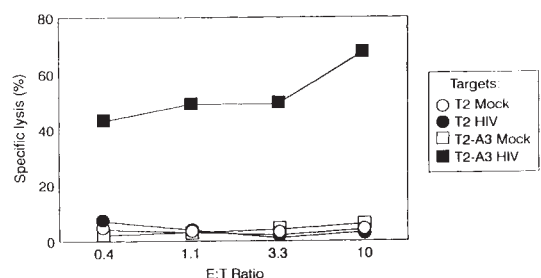
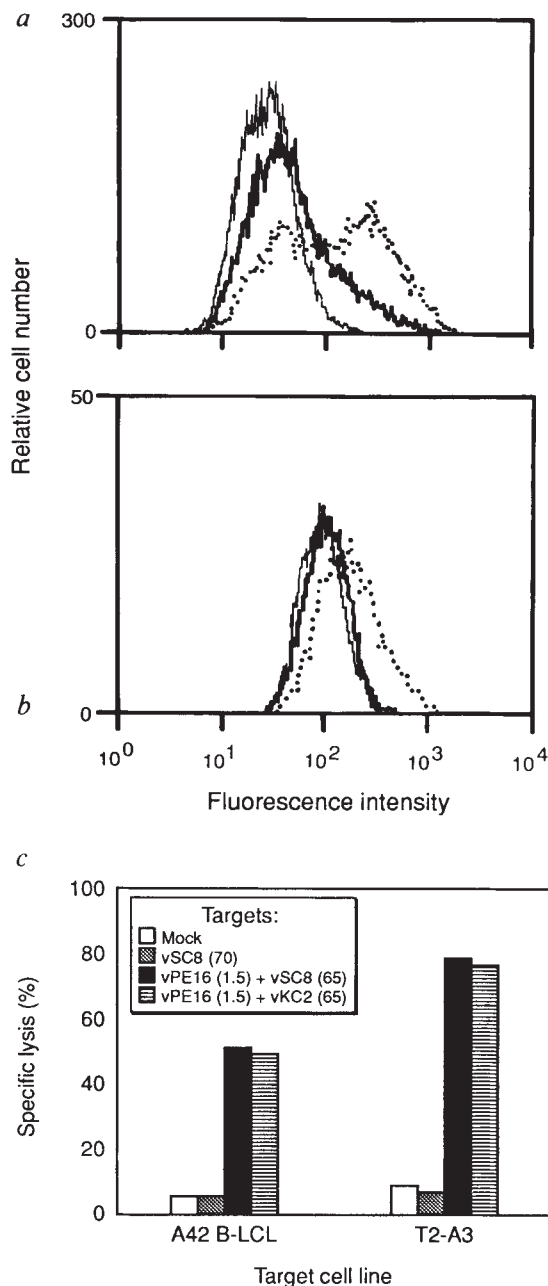


FIG. 3. Transporter-independent processing in HIV-1-infected cells. T2 and T2-A3 were infected with HIV-1 (LAI isolate) as previously described⁴⁷. Because T2 cells were derived by fusion of the mutant B-LCL LBL 721.174 with the CD4⁺ T-cell line CEM²¹, T2 and T2-A3 cells are CD4⁺ and can be readily infected with HIV-1. Five days after infection, mock- and HIV-1-infected T2 and T2-A3 cells were labelled with ⁵¹Cr and tested for susceptibility to lysis by clone A42.46.



(sCD4-KDEL) caused ER retention of env protein but had absolutely no effect on the percentage of transporter-competent A3.1⁺ B-LCL or transporter-defective T2-A3 cells lysed by env-specific CD8⁺ CTL clones (Fig. 4). Thus, transport of the env protein from the ER to the cell surface at the normal rate is not required for processing. Transporter-independent env processing is therefore unlikely to involve proteolysis of cell surface or shed env protein. Rather, the ER or a closely related compartment is likely to be the site of the reaction.

In summary, the HIV-1 env protein can be processed for recognition by CD8⁺ CTL by a Tap-1/Tap-2-independent mechanism that operates on env protein following its cotranslational translocation into the ER. Although some proteins, particularly misassembled subunits of oligomeric complexes, undergo degradation in the ER or a related compartment^{28,29}, the ER has not been considered a site at which peptides are generated for class I-restricted antigen recognition. Most naturally processed self peptides eluted from class I molecules are derived from abundant cytosolic and nuclear proteins, not proteins found in the ER or

FIG. 4 Retention of env protein in the ER does not affect class I processing. Retention was accomplished by coinfecting target cells with vPE16 and the vaccinia vector vKC2 which carries a truncated form of the CD4 gene in which sequences coding for the transmembrane and cytoplasmic domains are replaced with codons for the ER retention signal sequence Lys-Asp-Glu-Leu (sCD4-KDEL)⁴⁸. a, b, Flow cytometric analysis of the effects of sCD4-KDEL expression on cell surface expression of env protein. A42 B-LCL (a) or T2-A3 cells (b) were infected with the control vector vSC8 (thin line), with vPE16 and vSC8 (dotted line), or with vPE16 and vKC2 (thick line). After 10 h, cells were stained for surface expression of env protein and analyzed by flow cytometry. Analysis of env protein oligosaccharides in cells coexpressing env protein and sCD4-KDEL confirmed that although the env protein was synthesized in normal amounts in these cells, it was retained in the ER by sCD4-KDEL⁵⁰. Retention of env protein in the ER by sCD4-KDEL was also shown to block completely processing of env protein for class II-restricted recognition, a reaction that occurs after surface expression and internalization⁵⁰. c, Effect of sCD4-KDEL on lysis of A42 B-LCL and T2-A3 cells expressing the env gene. Target cells were infected as described above. Multiplicities of infection (MOI) are indicated in parentheses. After a 10 h incubation, target cells were tested for susceptibility to lysis by clone A42.46.

METHODS. The production of vKC2 is described in detail elsewhere⁵⁰. Infections were done at a constant total MOI of 68–70. vPE16 was used at an MOI of 3 and vKC2 at an MOI of 65. vSC8 was used as a control for vKC2 to give a constant total MOI for each sample. Staining of env protein on the surface of infected cells was done with the gp120-specific monoclonal antibody 902⁴⁹. Lysis was measured in a standard ⁵¹Cr release assay at an E:T ratio of 1:1.

the exocytic pathway³⁰. Transporter-defective cell lines have low cell surface expression of class I molecules, presumably because the cytosol is the principal source of peptides necessary for assembly and surface expression of stable class I molecules^{26,31–34}. T2 cells express a low level of surface HLA A2.1 molecules containing self peptides derived from the signal sequences of proteins that enter the exocytic pathway^{27,35}. The epitope recognized by the gp120-specific clones described here is located in mature gp120, downstream from the signal peptide cleavage site. Based on computer-assisted analysis of probable cleavage sites³⁶, it is unlikely to be generated by an aberrant downstream cleavage by signal peptidase.

It remains unclear whether other proteins that enter the exocytic pathway are subject to processing by this Tap-1/Tap-2-independent pathway. The transmembrane fusion protein of measles virus is processed by a Tap-1/Tap-2-dependent mechanism³⁷. However, some alloreactive CTL specific for conventional class I molecules and the class Ib Qa-1 molecules can lyse transporter-defective cells. The class I molecules recognized by these CTL may contain peptides generated in a transporter-independent fashion^{38,39}. The properties that enable the HIV-1 env protein (and possibly other proteins) to be processed by the Tap-1/Tap-2-independent pathway are not yet clear but may be related to the inefficient manner in which the env protein progresses through the exocytic pathway^{40,41} or its intracellular association with CD4⁴². Understanding of this novel Tap-1/Tap-2-independent processing pathway may aid the design of AIDS vaccines that will elicit env-specific CD8⁺ CTL. □

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1. Townsend, A. R. M., Gotch, F. M. & J. Davey *Cell* **42**, 457–467 (1985).
2. Gooding, L. R. & O'Connell, K. O. *J. Immunol.* **131**, 2580–2586 (1983).
3. Morrison, L. A., Lukacher, A. E., Braciale, V. L., Fau, D. & Braciale, T. J. *J. exp. Med.* **163**, 903–913 (1986).
4. Germain, R. N. *Nature* **322**, 687–689 (1986).
5. Townsend, A. R. M., Bastin, J., Gould, K. & Brownlee, G. G. *Nature* **324**, 575–577 (1986).
6. Moore, M., Carbone, F. R. & Bevan, M. J. *Cell* **54**, 777–785 (1988).
7. Yewdell, J. W., Bennick, J. R. & Hosaka, Y. *Science* **239**, 637–640 (1988).
8. Monaco, J. J. & McDermott, H. O. *Nature* **309**, 797–799 (1984).
9. Brown, M. G., Driscoll, J. & Monaco, J. J. *Nature* **353**, 355–357 (1991).
10. Glynn, R. et al. *Nature* **353**, 357–360 (1991).
11. Ortiz-Navarrete, V. et al. *Nature* **353**, 662–664 (1991).
12. Deverson, E. et al. *Nature* **348**, 738–741 (1990).
13. Trowsdale, J. et al. *Nature* **348**, 741–744 (1990).
14. Spies, T. et al. *Nature* **348**, 744–747 (1990).
15. Monaco, J. J., Cho, S. & Attaya, M. *Science* **250**, 1723–1726 (1990).

16. Kelly, A. et al. *Nature* **355**, 641–644 (1992).
17. Kleijmeier, M. J. et al. *Nature* **357**, 342–344 (1992).
18. Walker, B. D. et al. *Nature* **328**, 345–348 (1987).
19. Plata, F. et al. *Nature* **328**, 348–351 (1987).
20. Koenig, S. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8638–8642 (1988).
21. Salter, R. D., Howell, D. N. & Cresswell, P. *Immunogenetics* **21**, 235–246 (1985).
22. DeMars, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8183–8187 (1985).
23. Cerundolo, V. et al. *Nature* **345**, 449–452 (1990).
24. Hosken, N. A. & Bevan, M. J. *Science* **248**, 367–370 (1990).
25. Anderson, K. et al. *J. exp. Med.* **174**, 489–492 (1991).
26. Townsend, A. et al. *Nature* **340**, 443–448 (1989).
27. Wei, M. L. & Cresswell, P. *Nature* **356**, 443–446 (1992).
28. Lippincott-Schwartz, J., Bonifacio, J. S., Yuan, L. C. & Klausner, R. D. *Cell* **54**, 209–220 (1988).
29. Wilstrom, L. & Lodish, H. F. *J. Cell Biol.* **113**, 997–1007 (1991).
30. Jardeitzky, T. S., Lane, W. S., Robinson, R. A., Madden, D. R. & Wiley, D. C. *Nature* **353**, 326–329 (1991).
31. Lie, W.-R. et al. *Nature* **344**, 439–441. (1990).
32. Ljunggren, H.-G. et al. *Nature* **346**, 476–480 (1990).
33. Kvist, S. & Hamann, U. *Nature* **348**, 446–448 (1990).
34. Otten, G. R. et al. *J. Immunol.* **148**, 3723–3732 (1992).
35. Henderson, R. A. et al. *Science* **255**, 1264–1266 (1992).
36. Folz, R. J. & Gordon, J. I. *Biochem. biophys. Res. Commun.* **146**, 870–877 (1987).
37. van Binnendijk et al. *J. exp. Med.* **176**, 119–128 (1992).
38. Ohlen, C. et al. *J. Immunol.* **145**, 52–58 (1990).
39. Aldrich, C. J. et al. *J. Immunol.* **149**, 3773–3777 (1992).
40. Willey, R. L., Bonifacio, J. S., Potts, B. J., Martin, M. A. & Klausner, R. D. *Proc. natn. Acad. Sci. U.S.A.* **85**, 9580–9584 (1988).
41. Haffar, O. K., Nakamura, G. R. & Berman, P. W. *J. Virol.* **64**, 3100–3103 (1990).
42. Crise, B., Buonocore, L. & Rose, J. K. *J. Virol.* **64**, 5585–5593 (1990).
43. Earl, P. E., Hugin, A. W. & Moss, B. *J. Virol.* **64**, 2448–2451 (1990).
44. Earl, P. L., Koenig, S. & Moss, B. *J. Virol.* **65**, 31–41 (1991).
45. Polydefkis, M. et al. *J. exp. Med.* **171**, 875–887 (1990).
46. Hammond, S. A. et al. *J. exp. Med.* **176**, 1531–1542 (1992).
47. Orentas, R. J. et al. *Science* **248**, 1234–1237 (1990).
48. Buonocore, L. & Rose, J. K. *Nature* **345**, 625–628 (1990).
49. Chesebro, B. & Wehrly, K. J. *J. Virol.* **62**, 3779–3785 (1988).
50. Callahan, K. C. et al. *J. Immunol.* (in the press).

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***Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1**

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THE plant hormone auxin has a central role in many aspects of plant growth and development^{1,2}. By screening for mutants of *Arabidopsis* that are resistant to exogenous auxin, we have identified several genes that are required for normal auxin response³. One of these genes, *AXR1*, is defined by recessive mutations that confer auxin resistance to the roots, rosettes and inflorescences of mutant plants^{4–6}. In addition, *axr1* mutants display a variety of morphological defects that are consistent with a reduction in auxin sensitivity⁵. Here we isolate the *AXR1* gene using a map-based approach and report that *AXR1* encodes a new protein with significant sequence similarity to the ubiquitin-activating enzyme E1. The *AXR1* protein is highly diverged from previously characterized E1 enzymes, however, and lacks a key cysteine residue that is essential for E1 activity⁷. *AXR1* may therefore define a new class of enzymes in the ubiquitin pathway or it may have a novel function in cellular regulation which is unrelated to ubiquitin conjugation.

The *axr1* mutation has been mapped to the end of chromosome 1 (ref. 5). To obtain detailed information on the position of *axr1*, we analysed 126 chromosomes resulting from recombination between *axr1* and the proximal marker *dis1* for segregation of restriction-fragment length polymorphisms (RFLPs) detected by genomic clones 1a8, 488, and 6811 (Fig. 1a). With respect to *axr1*, eight chromosomes were recombinant for 1a8, one for 488 and none for 6811, indicating that the order of markers is 1a8–488–*axr1*, with approximately 0.1 centimorgans between 488 and *axr1*. One recombinant between *axr1* and the distal marker *cer1* was analysed and found to be recombinant for 6811 but not for 488 or 1a8, indicating that 6811 is distal to *axr1*. These results indicate that the *axr1* gene lies between genomic clones 6811 and 488.

A chromosome walk to *axr1* was initiated by probing a yeast

artificial chromosome (YAC) library of *Arabidopsis* genomic DNA⁸ with both 6811 and 488. One YAC, 14B10, hybridized to both probes, indicating that the insert in this YAC spanned the region between the two genomic clones (Fig. 1b). Cosmid clones spanning this region were generated in the pOCA18 (ref. 9) plant transformation vector. To identify the *AXR1* gene, each cosmid was introduced into *axr1*–3 plants by *Agrobacterium*-mediated transformation¹⁰. The progeny of individual transformants were scored for kanamycin resistance, auxin resistance and *axr1* morphology. Cosmids pOCA18-J and pOCA18-H restored wild-type auxin sensitivity (data not shown) and morphology to *axr1*–3 plants (Fig. 1c), indicating that both of these cosmids complemented the *axr1* mutation.

The pOCA18-H cosmid contains a 16.5-kilobase (kb) insert. Transcribed regions within this DNA were identified by probing RNA blots of poly(A)⁺ RNA isolated from wild-type seedlings and rosette leaves. Two transcripts were detected: a highly abundant 0.8-kb transcript and a less abundant 1.8-kb transcript (data not shown). The location of the two transcribed regions is shown in Fig. 3a. A complementary DNA library prepared from seedling RNA was screened with DNA fragments spanning the pOCA18-H insert. Two classes of cDNAs were recovered that corresponded to the two transcripts identified on RNA blots. To determine whether either of these classes of cDNAs represent the *AXR1* gene, RNA blot analysis was performed with RNA isolated from the mutants *axr1*–3, *axr1*–5, *axr1*–12, *axr1*–19, and *axr1*–23 (ref. 5). The 0.8-kb transcript was unaltered in size or abundance in any of the mutants compared to wild type (data not shown). In contrast, the levels of the 1.8-kb transcript were altered in each of the mutants except *axr1*–3, a weak allele (Fig. 2a). In addition, a smaller transcript was detected in *axr1*–23 RNA (Fig. 2b). Further analysis is required to determine the nature of this transcript. Because these data strongly suggest that the 1.8-kb transcript is the product of the *AXR1* gene, we determined the nucleotide sequences of a full-length cDNA, and of the corresponding genomic region. These results indicate that the gene includes 13 introns (Fig. 3b) and has a coding region that spans 1,620 nucleotides (Fig. 3c).

To confirm that this sequence represents the *AXR1* gene, we sequenced two mutant genes: *axr1*–3, a weak allele (Fig. 1c), and *axr1*–12, a strong allele. Genomic sequences from both mutants were amplified by polymerase chain reaction (PCR), cloned into a plasmid vector, and sequenced. PCR products from at least two independent reactions were analysed in each case. This analysis showed that the *axr1*–3 mutation is a missense mutation that replaces the cysteine at position 154 with a tyrosine. The *axr1*–12 mutation introduces a stop codon in exon 11 (Fig. 3c).

The *AXR1* gene encodes a protein of 540 amino acids with an approximate relative molecular mass (M_r) of 60,000 (Fig. 3c). Comparison of the deduced amino-acid sequence of *AXR1* with protein sequences in the database reveals significant sequence

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FIG. 1 a, Position of the *AXR1* locus on chromosome 1. Map distances between *axr1* and the markers *cer1* and *dis1* were determined using F₂ segregation data and the Linkage 1 program¹⁹. The relative position of *axr1* and the genomic clones 488, 1a8 and 6811 (refs 20, 21) was established by examining segregation of RFLPs in plants with recombination breakpoints in either the *dis1-axr1* or *axr1-cer1* intervals. b, Map of the *AXR1* region between the proximal end of genomic clone 488 and the distal end of genomic clone 6811. Clone 8354 is the most distal member of a contiguous series of cosmids (isolated and mapped by B. Hauge, personal communication). Six YAC clones, 12G9, 18E6, 2H9, 12E8, 14B10 and 8E8, were isolated from an *Arabidopsis* genomic library constructed by Ward and Jen⁸, using 6811 and 488 as probes. Seven contiguous clones, E, I, D, C, B, J and H, were isolated in the pOCA18 binary T-DNA cosmid vector⁹.

Cosmids B and H were constructed by subcloning 6811 DNA into the pOCA18 vector. Cosmids E, I, D, C and J were isolated by screening pOCA18 *Arabidopsis* genomic libraries⁹. The probes used to recover these cosmids were prepared from clones 488 and 8354, a 0.4-kb restriction fragment derived from the most proximal end of H, and the subcloned left end of YAC 18E6. c, Photograph of 7-week-old *Arabidopsis* plants. From left to right the plants are: wild-type, *axr1-3* and *axr1-3* transformed with pOCA18-H. Similar results were obtained with multiple transformants from three independent experiments.

METHODS. Plants used for RFLP analysis were recovered from an F₂ population generated by a cross of *axr1* (Col-O) with *dis1*, *cer1* (Ler). Recombinant plants that were *axr1*, *dis1* or *axr1*, *cer1* were identified and used to establish F₃ families. DNA was isolated from F₃ plants²², digested with the appropriate restriction enzyme and electrophoresed in agarose gels. DNA was blotted onto Hybond-NTM (Amersham) according to the manufacturers instructions. Probes were prepared by oligo-labelling. Hybridization and washing were done under standard conditions²³. Both YAC and cosmid libraries were screened using standard procedures²³. The pOCA18 library was obtained from N. Olszewski (University of Minnesota) and the YAC library from E. Ward (Ciba Geigy). Seedlings were grown in a 16-hour-light, 8-hour-dark cycle at 22 °C on standard agar medium⁵. *Agrobacterium*-mediated transformation was as described¹⁰, except that the media were modified to optimize transformation of *axr1* mutants. Callus-inducing medium contained 20 mM 2,4-D and shoot-inducing medium contained 0.5 µM BA and 2 µM NAA.

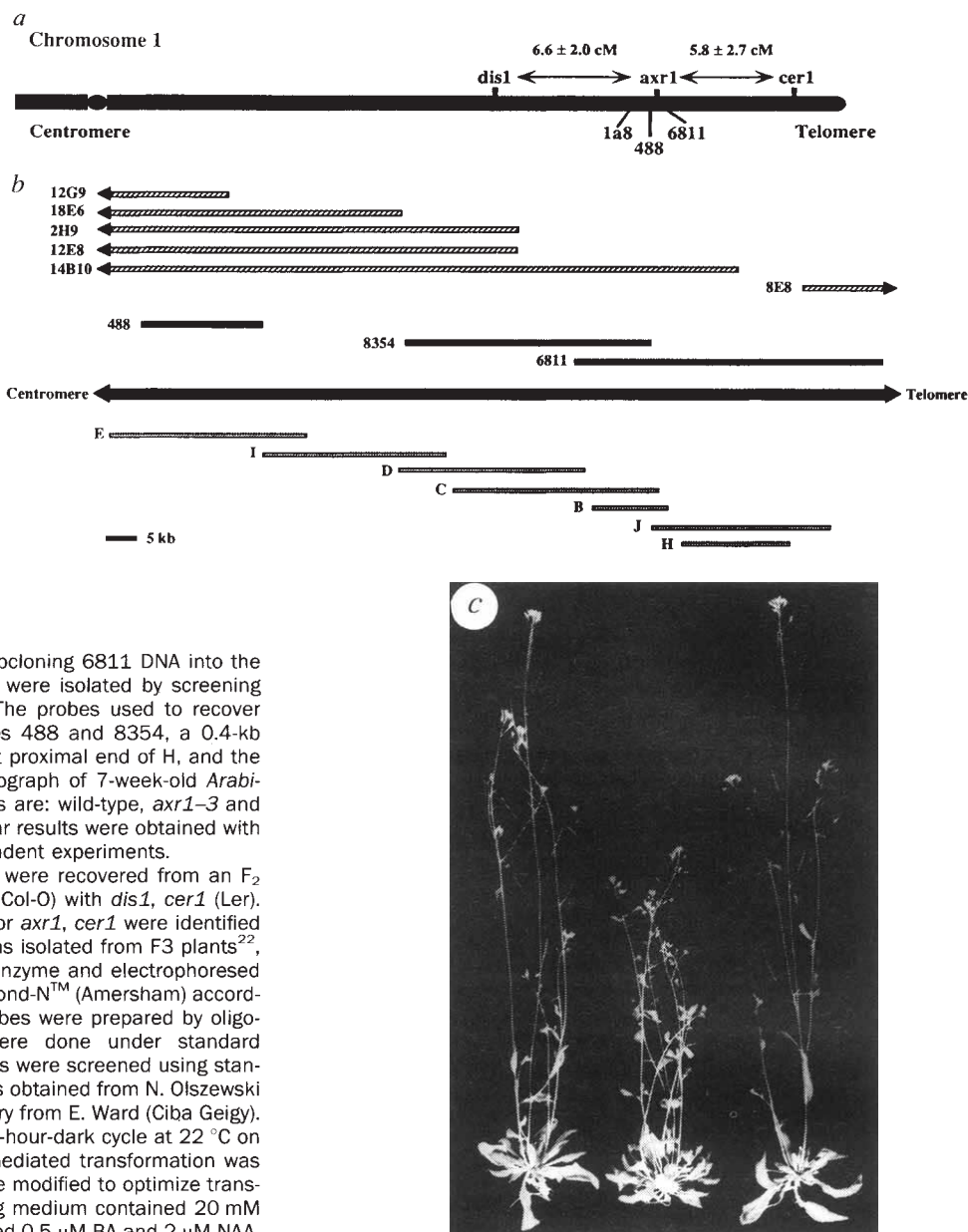
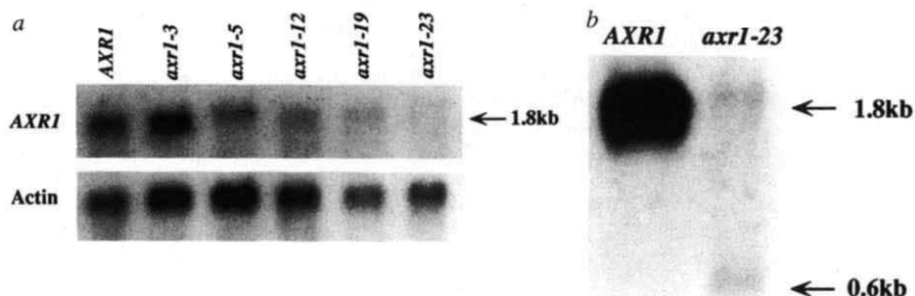


FIG. 2 a, Top panel shows an RNA gel blot of poly(A)⁺ RNA extracted from rosette tissue of wild-type and mutant plants and hybridized to an *AXR1* cDNA clone. The *axr1-3*, *axr1-5*, *axr1-12* and *axr1-19* mutants were recovered from ethylmethane sulphonate (EMS)-treated populations; *axr1-23* was recovered after treatment with γ -rays⁵. Each allele confers a similar severe phenotype except *axr1-3*, which is a weak allele. Bottom panel shows the same blot stripped and reprobed with a genomic clone of an *Arabidopsis* actin gene²⁴ to demonstrate equal RNA loading in all lanes (actin clone from R. Ferl). b, RNA gel blot of poly(A)⁺ RNA from rosette tissue of wild-type and *axr1-23* plants probed with a full-length *AXR1* cDNA clone.

METHODS. Total RNA was extracted from frozen rosette tissue by a method adapted from ref. 25. Poly(A)⁺ RNA was isolated using a Strata-gene Poly(A) Quik Kit according to the manufacturer's instructions and electrophoresed on 1% agarose gels containing 2.2 M formaldehyde²³, transferred onto Hybond N membrane and probed with a ³²P-labelled DNA probe according to the manufacturer's instructions. Probes were prepared using random oligonucleotide primers. RNA size was estimated using M_r standards (BRL).



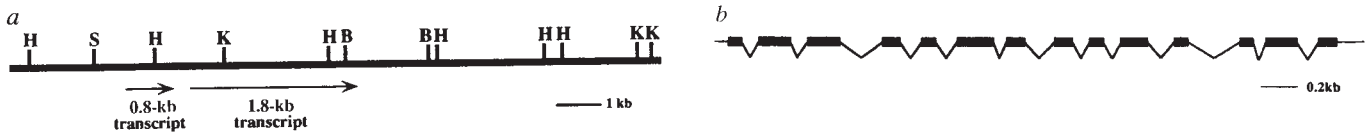


Fig. 3 a, Map of the pOCA18-H insert DNA. The positions of 2 transcripts produced from this region are shown and their direction of transcription indicated by arrows. Transcripts were mapped by probing RNA blots with specific restriction fragments derived from pOCA18-H. cDNA clones corresponding to both these transcripts were obtained by probing a seedling-derived *Arabidopsis* cDNA library with pOCA18-H. The location of transcribed regions was confirmed by hybridizing Southern blots of pOCA18-H DNA digested with restriction enzymes to probes prepared from each cDNA clone. The direction of transcription was established by sequencing cDNA clones. H, *Hind*III; S, *Sal*I; K, *Kpn*I; B, *Bgl*II. b, Structure of the AXR1 gene. Exons are denoted by thick lines and introns by thin lines. Exon boundaries were determined by comparison of cDNA sequence and genomic sequence. The genomic sequence spans 3,388 nucleotides (including introns) and has been established on both strands. The introns range in size from 88 to 309 nucleotides. The sequence has been submitted to the Genbank (accession number L13922). c, Nucleotide and predicted amino-acid sequence (single-letter code) of AXR1. Nucleotides are numbered on the left, starting with the first nucleotide of the initiating AUG. Amino acids are numbered in italics on the right, beginning at the first predicted methionine. The positions of the *axr1-3* and *axr1-12* mutations are indicated in bold; *axr1-3* is a G → A at nucleotide 461 and *axr1-12* is a C → T at nucleotide 1,246.

METHODS. Southern analysis was done as described in Fig. 1 legend. RNA isolation and RNA blot analysis have been described in the legend to Fig. 2. cDNA clones were recovered from a λ ZAP II (Stratagene) cDNA library prepared from seedling RNA by J. Ecker (University of Pennsylvania). Subclones for sequencing were generated by excision of pBluescript phagemids from λ ZAP II clones. Double-stranded plasmids containing either cDNA or genomic subclones were used to determine DNA sequence using Sequenase version 2.0 (USB), as described by the manufacturer. Sequences were compiled using GCG programs¹¹.

Fig. 4 Alignment of amino-acid sequences of AXR1 with the N-terminal regions of ubiquitin-activating enzyme (E1) from human¹², yeast¹³ and wheat¹⁴. Amino-acid sequence alignment was produced by the PileUp routine of the Wisconsin GCG software package¹¹. Residues that are conserved between AXR1 and at least one of the E1 sequences are displayed as white letters in black boxes. The line below the sequences indicates the region of highest similarity between AXR1 and E1. The total length of the E1 enzymes are 1,058 (human), 1,024 (yeast) and 1,051 (wheat) amino acids.

human	M S S S P L S K K R	R V S G P D P K P G	S N C S P A O S V L	S E V P S V P T N G	M A K N G S E A D I	D E G L V S R O L V	60
yeast							24
wheat							53
AXR1							29
human	V L G E A M K R L	Q T S V L V S G L	R G L G V E I A K N	I L L G G V K A V T	L H D G G T A Q W A	D L S S Q F Y L R E	120
yeast	V L G E A M K R L	Q T S V L V S G L	R G L G V E I A K N	I L L G G V K A V T	L H D G G T A Q W A	D L S S Q F Y L R E	84
wheat	V L G E A M K R L	Q T S V L V S G L	R G L G V E I A K N	I L L G G V K A V T	L H D G G T A Q W A	D L S S Q F Y L R E	113
AXR1	I W G E V G O A A L	E E A S I C L L N C	G P T G S E A L K N	L V L G G V G S I T	V D G S K V O F G	D L G N N F M V D A	89
human	E D I K C N R A E V	S Q P R A E L N S	V P V T A Y T G P	L V E D	F L S G F Q V V V L	T N T P L E D O L	173
yeast	N D I G C K R G D V	T R A K A E L N A	V P V N V L D S L	D V T	Q L S G F Q V V V L	T D P S L E D K V	138
wheat	N D V G N R R A Q A	C V O K Q E L N N	A V L V S A L T G D	U T K E	H L S K F O A V V F T	T D P L S L O K A	165
AXR1	K S V G G S K A K S	K A F Q E L N D	S V N A K F I E E N	P D L T I T N P S	F S Q F T L V I A	T O L V E D S M L	148
human	R V G E F C H N .	R G I K L V A D T	R G L F G Q L .	... F C D F G E E	M I L T D S N G E Q	P L S A M V S M V T	225
yeast	X I N E R C H S .	S G I R F I S S E T	R G L F G N T .	... F V D L G D E	F T V L O P T G E E	P R T C M V S D I E	190
wheat	E F D D V C H S Q Q	P P I A F I K S E V	R G L F G S V .	... F C D F G E E	F T V L D V D G E E	P H T G I V A S I T	220
AXR1	K L D R I C R D A N	V K L V L V R S Y G	L A G F V R I S V K	E H P I D S K D E	H F L D D L R L N N	P W F E L K S F V E	208
human	K D N P G V .	... V T C L D E A R H	G F E S G D F V S .	... F V D L G D E	F T V L O P T G E E	P R T C M V S D I E	249
yeast	P D . G T .	... V T M L D D N R H	G L E D G N F V R .	... F V D L G D E	F T V L O P T G E E	P R T C M V S D I E	244
wheat	N D N P A L .	... V S C V D E L R E	E T Q D D E V V P .	... F C D F G E E	F T V L D V D G E E	P H T G I V A S I T	268
AXR1	T I D L N V S E P A	A A H K I P Y V V	I L V K A L E W A	Q S H S G N L F S T	R E E K K E F K D L	V K S K M V S T D E	248
human	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	271
yeast	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	244
wheat	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	266
AXR1	D N Y K E A I E A A	F K V F A P R G I S	S E V Q K L I N D S	C A E V N S N S S A	F W Y M V A A L K E	F V L N E G G G A	328
human	P Y T F S . C D T	S N F S O V .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	294
yeast	P F A F R I . G S V	K E Y G E V .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	267
wheat	P Y S F L E E D T	S S F G A V .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	... F S E V Q G M V E .	290
AXR1	P L E G E T P D M T	S S T E R V L Q	K I Y L A K A E A D	F L V I E E R V K N	I L K K I G R D P S	S I P K P T I K S F	388
human	V K V P K K I S .	... F K S L V A S L A	E P . D F V V T D F .	... A K F E R P A	Q L H I G F O A L H	Q F C A Q H . G R P	346
yeast	V K V P K K I S .	... F K S L K Q O L S	N P . E F V F S D F .	... A K F D R P A	Q L H L G F O A L H	Q F V Y H N G E	320
wheat	V K P P V I K .	... F K P L K E A M S	E P G E F L M S D F .	... S K F E R P P	L L W L A F O A L H	K R T E L S R F	343
AXR1	C K N A K L K L C	R Y R M V E D E F R	N P S V T E I Q K Y	L A D E D Y S O A M	G F Y I L L R A A D	R F A A . N Y N K F	447
human	P R P R N E E D A A	E L V A . A G A V N	A R A L P A V Q O N	... N L D E D L R K	L A Y V A A G D L A	P I N A F I G G L A	405
yeast	P R T M N D E A D A	E L I K V . I D L S	V Q Q P E V L G E Q	... V O V N D E L K E	L S Y G A R G O P	G V Y A F F G G L V	380
wheat	P V A G S T D D V Q	R V I E Y . A I S	I N D P T L G R K L	... E I D K L E R H	F A S G S R A V L H	P K A R N F G G I V	401
AXR1	P G Q F D G G M D E	D I S R K L T A L	S L L T D L G C N G	S V L P D O L L E R	M C R F G A S E I H	V S A F V G G I A	507
human	A Q E V M K A C S G	K F M P I N Q W L Y	... D A L E C L P E D	... K E V L T E D K C L	Q R O N R	I A V F G L O F Q K	450
yeast	A Q E V M K A C S G	K F T P L K Q F M Y	... R D S L E S L P D P	... K N F P R N E K T T	Q P V N S R Y D A Q	I A V F G L O F Q K	440
wheat	G O E V M K A C S G	K F H P L Y Q F F Y	... R D S V E S L P D P	... P L E P G D L	K P N S R Y D A Q	I A V F G L O F Q K	450
AXR1	S O E V M K L V T K	Q F V P M L G T Y I	... A N G I D H K S Q L	... L K L	...	...	540

similarity to ubiquitin-activating enzyme (E1) (ref. 11). E1 is a highly conserved enzyme of M_r 105 to 120K, depending on the species^{12–14}. The AXR1 protein has regions of sequence similarity within the N-terminal 450 amino acids of E1. An alignment of AXR1 with the N terminus of E1 from human, yeast and wheat is shown in Fig. 4. Sequence similarity is highest in a 60-amino-acid stretch near the N terminus of both AXR1 and E1. Two genes encoding E1 from *Arabidopsis* have also been cloned and sequenced. The products of these genes are similar to the other E1 proteins that have been described (P.M. Hatfield and R.D. Vierstra, personal communication).

The E1 enzyme functions in the biosynthesis of ubiquitin-protein conjugates by catalysing the formation of a thiol ester bond between the C terminus of ubiquitin and a cysteine residue within the E1 protein¹⁵. The ubiquitin molecule is then transferred to one of a family of ubiquitin-conjugating enzymes (E2) (ref. 15). E2 in turn transfers ubiquitin to a specific target protein. In many instances, ubiquitinated proteins are rapidly degraded by a specific ubiquitin-dependent protease complex¹⁵, but several stable ubiquitin conjugates have been described, suggesting that ubiquitination may have additional functions¹⁵. At present, specific biochemical functions have not been localized to the regions of similarity between AXR1 and E1. Although AXR1 may have E1 activity, a comparison of the two proteins suggests that this is unlikely. AXR1 is roughly half the length of E1 and numerous gaps are required to align the AXR1 and E1 sequences. Mutagenesis experiments have shown that a conserved cysteine at position 626 in wheat E1 is absolutely required for activity⁷. The region of similarity between AXR1 and E1 does not include residue 626. Two consensus ATP-binding domains have also been identified in yeast E1 (ref. 13) and neither of these domains is present in AXR1. The AXR1 protein has roughly the same level of similarity (22 to 27%, gaps excluded) to the wheat, yeast and human E1 enzymes, whereas pairwise similarities between the three E1 enzymes are much higher (45 to 53%)¹³. These results strongly indicate that AXR1 and E1 result from a duplication that occurred well over one billion (10⁹) years ago, the estimated time of divergence of plants, fungi and animals. During this divergence, AXR1 could have assumed a very different cellular function.

Despite these differences, the nature of the *axr1-3* mutation suggests that E1 and AXR1 are functionally related. This mutation substitutes tyrosine for cysteine at position 154, a residue that is conserved between AXR1 and all E1 enzymes so far characterized. In wheat E1, changing this cysteine to serine results in a dramatic decrease in enzyme activity. Thus this cysteine residue has an important and possibly similar function in both AXR1 and the E1 enzymes⁷.

The similarity between AXR1 and E1 suggests that the ubiquitin pathway may have an important role in plant hormone action. Studies in vertebrate cells indicate that several membrane receptors are ubiquitinated in response to ligand binding^{16–18}. In the case of the IgE receptor, ubiquitination is rapidly reversible upon ligand disassociation, suggesting that ubiquitination may have a regulatory function¹⁸. By analogy, the function of an auxin receptor might be altered by ubiquitination. Alternatively, the cellular concentration of a protein(s) that mediates auxin response may be regulated by ubiquitin-mediated proteolysis. □

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- Evans, M. L. in *Encyclopedia of Plant Physiology* **10** (ed. Scott, T. K.) 23–79 (1985).
- Davies, P. J. in *Plant Hormones and their Role in Plant Growth and Development* (ed. Davies, P. J.) 1–11 (1988).
- Estelle, M. *BioEssays* **14**, 439–444 (1992).
- Estelle, M. & Somerville, C. R. *Molec. gen. Genet.* **206**, 200–206 (1987).
- Lincoln, C. et al. *Plant Cell* **2**, 1071–1080 (1990).
- Lincoln, C. & Estelle, M. *Iowa Acad. Sci.* **98**, 68–71 (1991).
- Hatfield, P. M. & Vierstra, R. D. *J. biol. Chem.* **267**, 14799–14803 (1992).
- Ward, E. R. & Jen, G. C. *Plant molec. Biol.* **14**, 561–568 (1990).
- Oliszewski, N. E. et al. *Nucleic Acids Res.* **16**, 10765–10782 (1988).
- Valvekens, D. et al. *Proc. natn. Acad. Sci.* **85**, 5536–5540 (1988).
- Devereaux, J., Haeberli, P. & Smithies, O. *Nucleic Acids Res.* **12**, 387–394 (1984).
- Handley, P. M. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 258–262 (1991).

- McGrath, J. P., Jentsch, S. & Varshavsky, A. *EMBO J.* **10**, 227–236 (1991).
- Hatfield, P. M., Callis, J. & Vierstra, R. J. *biol. Chem.* **265**, 15813–15817 (1990).
- Finley, K. & Chau, V. A. *Rev. Cell Biol.* **7**, 25–69 (1991).
- Cenciarelli, C. et al. *Science* **257**, 795–797 (1992).
- Mori, S., Heldin, C.-H. & Claesson-Welsh, L. *J. biol. Chem.* **267**, 6429–6434 (1992).
- Paolini, R. & Kinet, J.-P. *EMBO J.* **12**, 779–786 (1993).
- Suiter, K. A., Wendel, J. F. & Case, J. S. *J. Heredity* **74**, 203–204 (1983).
- Chang, C. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6856–6860 (1983).
- Nam, H.-G. et al. *Plant Cell* **1**, 699–705 (1989).
- Dellaporta, S. L. et al. *Plant molec. Biol. Rep.* **1**, 181–192 (1983).
- Sambrook, J. et al. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, New York, 1989).
- Nairn, C. J. et al. *Gene* **85**, 247–257 (1988).
- Puissant, C. & Houdebine, L.-M. *Biotechniques* **8**, 148–149 (1990).

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Universal nucleic acid-binding domain revealed by crystal structure of the *B. subtilis* major cold-shock protein

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THE cold-shock response in both *Escherichia coli* and *Bacillus subtilis* is induced by an abrupt downshift in growth temperature. It leads to the increased production of the major cold-shock proteins, CS7.4 and CspB, respectively^{1–3}. CS7.4 is a transcriptional activator of two genes^{4,5}. CS7.4 and CspB share 43 per cent sequence identity with the nucleic acid-binding domain of the eukaryotic gene-regulatory Y-box factors⁶. This cold-shock domain is conserved from bacteria to man⁷ and contains the RNA-binding RNP1 sequence motif⁸. As a prototype of the cold-shock domain, the structure of CspB has been determined here from two crystal forms. In both, CspB is present as an antiparallel five-stranded β -barrel. Three consecutive β -strands, the central one containing the RNP1 motif, create a surface rich in aromatic and basic residues that are presumably involved in nucleic acid binding. Preferential binding of CspB to single-stranded DNA is observed in gel retardation experiments.

In both crystal forms (Table 1), CspB is present as a compact molecule with overall dimensions of $30 \times 30 \times 30 \text{ \AA}^3$ and is composed entirely of antiparallel β -sheet with connecting turns and loops. CspB contains two subdomains, the first consisting of strands $\beta 1$ to $\beta 3$ and a loop of 16 residues (Fig. 1a, b). The second consists of strands $\beta 4$ and $\beta 5$ and is packed against the first at an angle of approximately 90° to give the molecule an asymmetric L-shape. Strand $\beta 2$ of subdomain 1 contains the RNP1 sequence motif found in a variety of RNA-binding proteins⁸. Subdomain 2 is packed against subdomain 1 to form a five-stranded β -barrel with a hydrophobic core, including residues Leu 2, Val 6, Phe 9, Ile 18, Val 20, Val 26, Val 28, Ile 33, Leu 41, Val 47, Phe 49, Ile 51 and Val 63, with one end protruding out of the β -barrel by $15 \text{ \AA}$. Strands $\beta 1$ to $\beta 4$ of the barrel form the well-known Greek key motif⁹ (Fig. 1c). The folding of CspB as seen in the crystal is very similar to that in solution as determined by NMR spectroscopy¹⁰.

Along the crystallographic 2-fold axis present in space group $P3_221$, two CspB molecules form a dimer. The dimer is stabilized

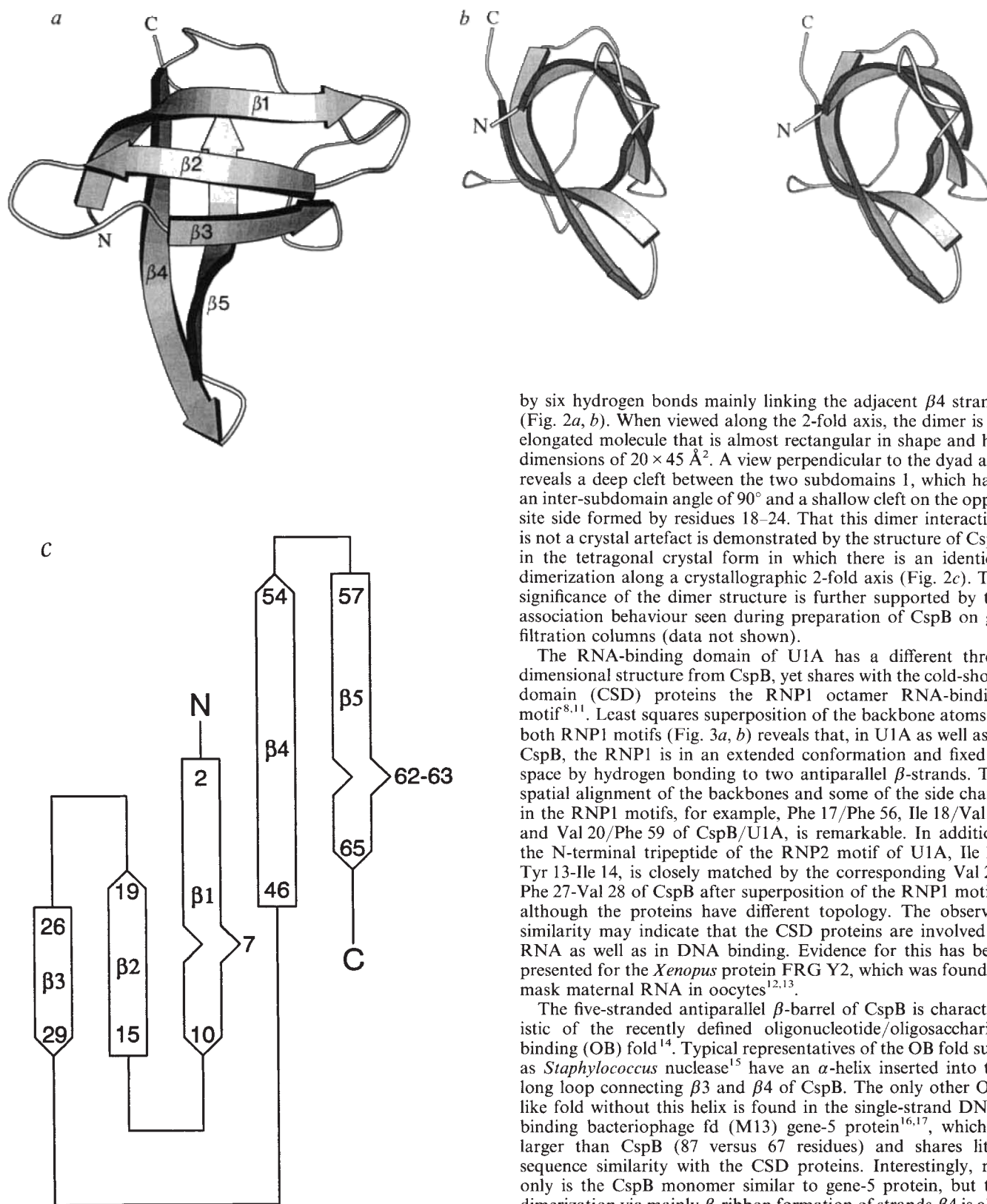


FIG. 1 Schematic drawing of the molecular structure of CspB. The β -strands are given as curved arrows. *b*, Schematic stereo drawing of CspB in an orientation that emphasizes the barrel structure and the core filled with hydrophobic residues (not shown). MOLSCRIPT was used to draw *a* and *b* as well as Figs 2 and 3*b*, *c* (ref. 27). *c*, Secondary structure of CspB according to ref. 28. Bulges at residues 7 and 62, 63 are indicated.

by six hydrogen bonds mainly linking the adjacent $\beta 4$ strands (Fig. 2*a, b*). When viewed along the 2-fold axis, the dimer is an elongated molecule that is almost rectangular in shape and has dimensions of $20 \times 45 \text{ \AA}^2$. A view perpendicular to the dyad axis reveals a deep cleft between the two subdomains 1, which have an inter-subdomain angle of 90° and a shallow cleft on the opposite side formed by residues 18–24. That this dimer interaction is not a crystal artefact is demonstrated by the structure of CspB in the tetragonal crystal form in which there is an identical dimerization along a crystallographic 2-fold axis (Fig. 2*c*). The significance of the dimer structure is further supported by the association behaviour seen during preparation of CspB on gel filtration columns (data not shown).

The RNA-binding domain of U1A has a different three-dimensional structure from CspB, yet shares with the cold-shock domain (CSD) proteins the RNP1 octamer RNA-binding motif^{8,11}. Least squares superposition of the backbone atoms of both RNP1 motifs (Fig. 3*a, b*) reveals that, in U1A as well as in CspB, the RNP1 is in an extended conformation and fixed in space by hydrogen bonding to two antiparallel β -strands. The spatial alignment of the backbones and some of the side chains in the RNP1 motifs, for example, Phe 17/Phe 56, Ile 18/Val 57 and Val 20/Phe 59 of CspB/U1A, is remarkable. In addition, the N-terminal tripeptide of the RNP2 motif of U1A, Ile 12-Tyr 13-Ile 14, is closely matched by the corresponding Val 26-Phe 27-Val 28 of CspB after superposition of the RNP1 motifs, although the proteins have different topology. The observed similarity may indicate that the CSD proteins are involved in RNA as well as in DNA binding. Evidence for this has been presented for the *Xenopus* protein FRG Y2, which was found to mask maternal RNA in oocytes^{12,13}.

The five-stranded antiparallel β -barrel of CspB is characteristic of the recently defined oligonucleotide/oligosaccharide binding (OB) fold¹⁴. Typical representatives of the OB fold such as *Staphylococcus* nuclease¹⁵ have an α -helix inserted into the long loop connecting $\beta 3$ and $\beta 4$ of CspB. The only other OB-like fold without this helix is found in the single-strand DNA-binding bacteriophage fd (M13) gene-5 protein^{16,17}, which is larger than CspB (87 versus 67 residues) and shares little sequence similarity with the CSD proteins. Interestingly, not only is the CspB monomer similar to gene-5 protein, but the dimerization via mainly β -ribbon formation of strands $\beta 4$ is also equivalent in both proteins. The topological similarity with gene-5 protein and the OB fold raises the question of whether the CSD also binds single-stranded DNA.

To bind to nucleic acids, the acidic CspB has to overcome the charge repulsion that should prohibit the approach of two negatively charged macromolecules. Positive charges are arranged mainly on one face of the CspB molecule, creating an attractive potential for nucleic acids (Fig. 3*c*). On this side of the molecule, together with Lys 7, Lys 13, His 29 and Arg 56, we

find the solvent-exposed aromatic side chains of Trp 8, Phe 15, Phe 17, Phe 27 and Phe 30. Phenylalanines 15, 17 and 27 of CspB are part of the RNP1 and RNP2 motifs. The phenylalanine residues 16 and 58 from the RNP1 and RNP2 motifs of the human A1 heterogeneous nuclear ribonucleoprotein, structurally corresponding to Phe 17 and Phe 27 of CspB, have been photochemically crosslinked to DNA¹⁸. The importance of this protein surface for nucleic acid binding has been demonstrated by replacement of the two phenylalanines within the RNP1 motif of the *E. coli* Rho protein with leucine and alanine, resulting in reduced affinity for RNA¹⁹. This may mean that CspB uses these

aromatic and basic side chains, which are conserved in the CSD proteins and correspond roughly to the common oligomer-binding site in the OB-fold proteins, for binding nucleic acid single strands. The exact mode of binding of CspB to DNA or RNA single strands cannot be predicted without knowledge of the molecular structure of the nucleic acid target, but it is clear that the surface region identified is ideally suited for this purpose. How CspB would bind to double-stranded DNA is less obvious from the crystal structure.

Gel retardation experiments have been carried out to assay DNA binding by CspB to the 35-base-pair double strand, as well

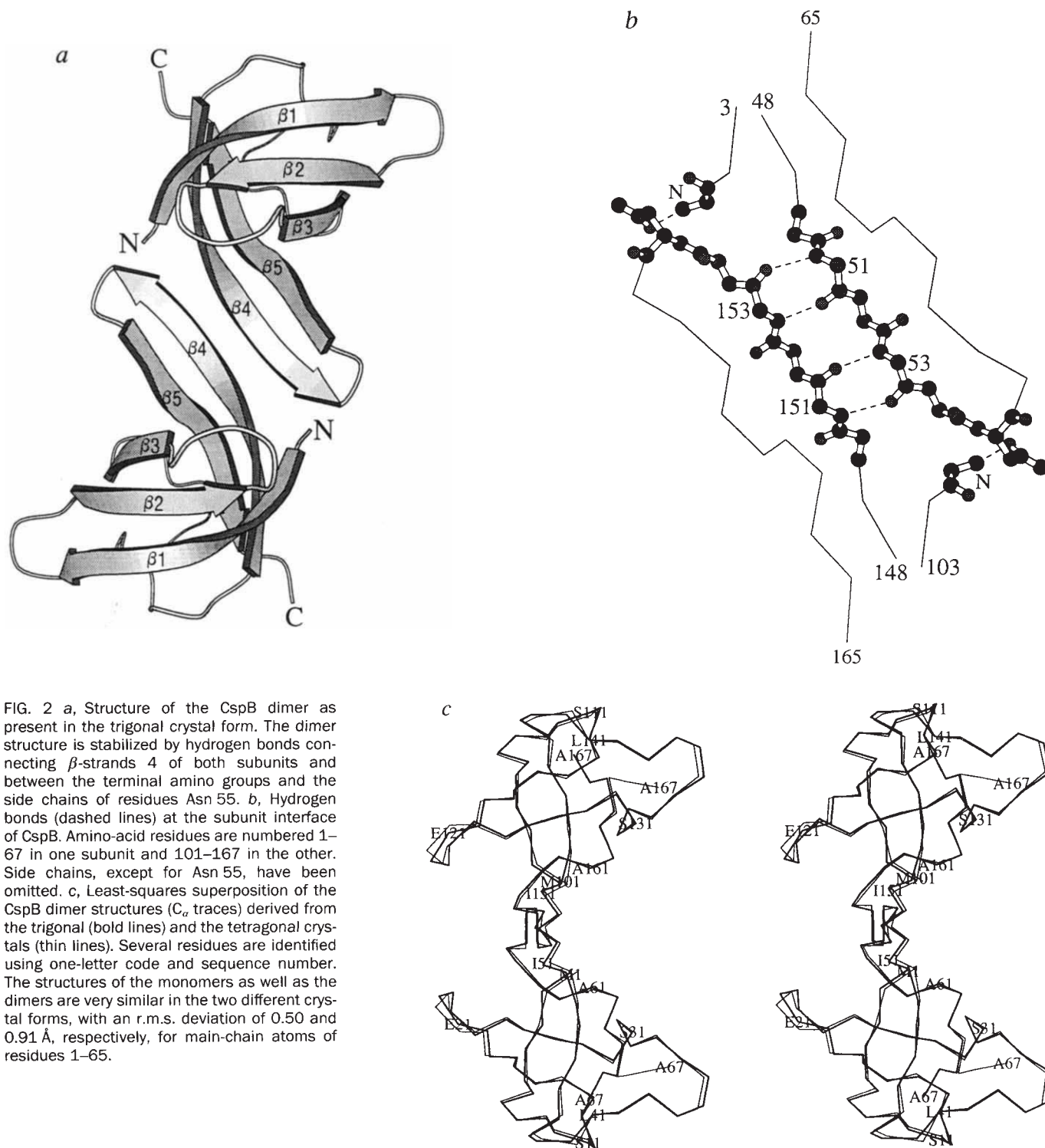
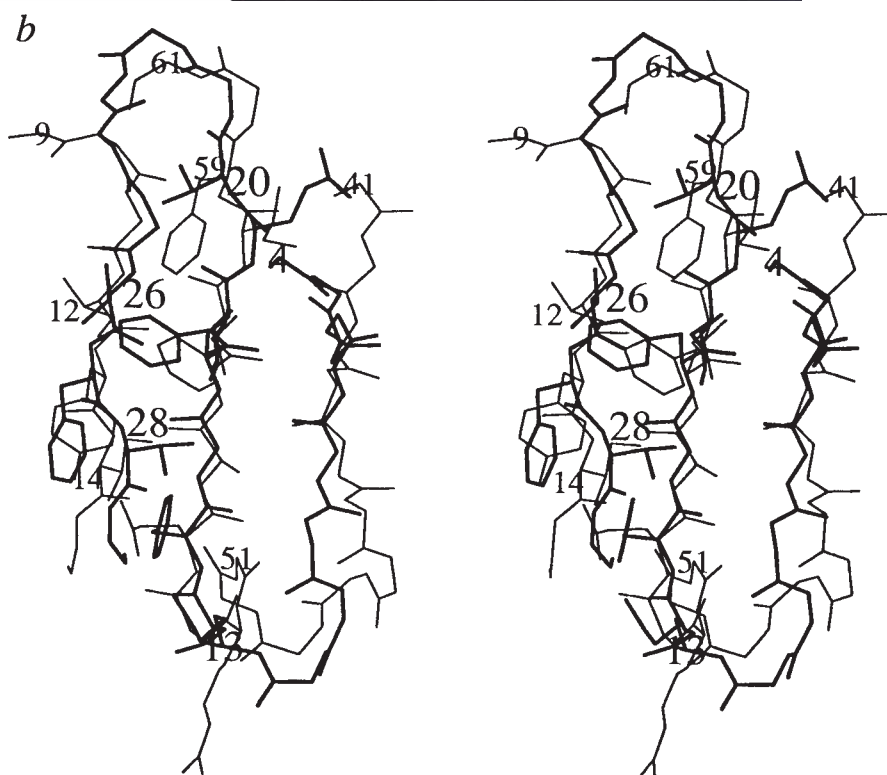
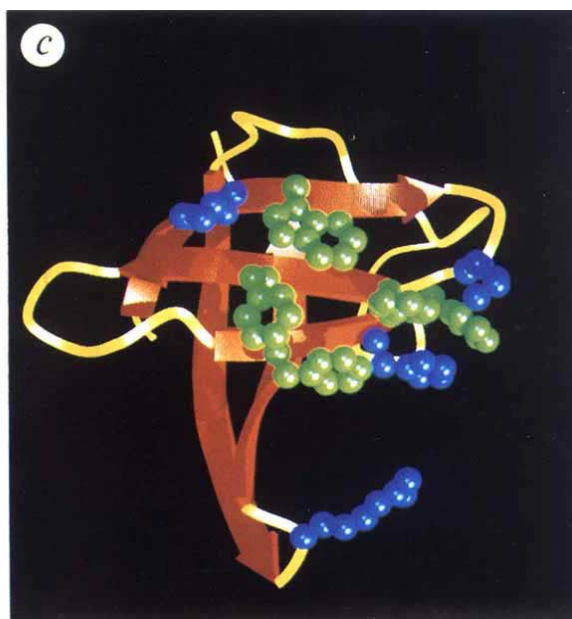
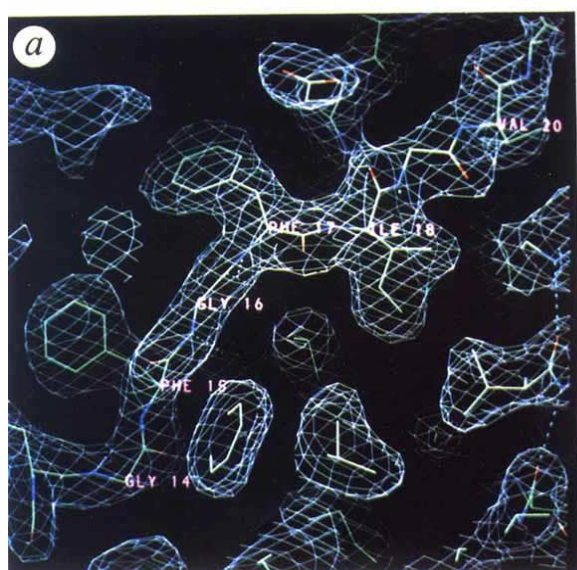


FIG. 2 *a*, Structure of the CspB dimer as present in the trigonal crystal form. The dimer structure is stabilized by hydrogen bonds connecting β -strands 4 of both subunits and between the terminal amino groups and the side chains of residues Asn 55. *b*, Hydrogen bonds (dashed lines) at the subunit interface of CspB. Amino-acid residues are numbered 1–67 in one subunit and 101–167 in the other. Side chains, except for Asn 55, have been omitted. *c*, Least-squares superposition of the CspB dimer structures (C_{α} traces) derived from the trigonal (bold lines) and the tetragonal crystals (thin lines). Several residues are identified using one-letter code and sequence number. The structures of the monomers as well as the dimers are very similar in the two different crystal forms, with an r.m.s. deviation of 0.50 and 0.91 Å, respectively, for main-chain atoms of residues 1–65.



d

DNA	ss35(+)					ds35					ss35(-)						
CspB:DNA	0	1	2	5	10	20	0	2	5	10	20	0	1	2	5	10	20

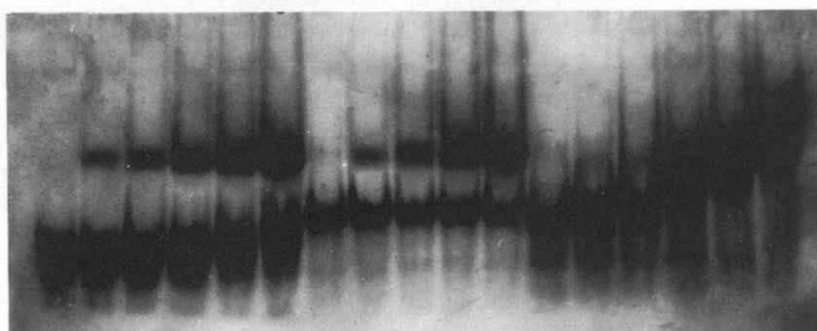


FIG. 3 **a**, Electron density ($2F_o - F_c$) of the crystal in space group $P3_221$ showing part of the RNP1 sequence motif. The map is contoured at 1.25 times the r.m.s. density and superimposed with the refined protein model. **b**, Least-squares superposition of the RNP1 motifs of CspB (bold lines) and the U1A (thin lines) with the two neighbouring strands. The backbone atoms of the octamer RNP1 motifs (CspB, KGFGFIEV; U1A, RGQAFVIF) superimpose with an r.m.s. difference between 32 equivalent main chain atoms of 1.07 Å. Side chains are displayed in the RNP1 and (partial) RNP2 motifs only. **c**, Aromatic (green) and basic (blue) residues on the presumed nucleic acid-binding surface of CspB. **d**, DNA-binding by CspB assayed by polyacrylamide gel retardation²⁹ of the 35-mer oligonucleotide 5'ACG-AGTATATCAGGCATTGGATGTGAATA - AAGCGT3' (plus strand). The complementary strand is centred about a CCAAT motif (position underlined). *E. coli* CS7.4 requires this motif for binding to the *gyrA* promoter⁵. 54 pmol each of the double strand (ds35) as well as the two single strands (ss35(+)) and ss35(-)) were incubated with CspB in 50 mM Tris-HCl, pH 8, 75 mM NaCl, 3 mM MgCl₂, 5% glycerol (by volume) at room temperature. The gel contained 10% polyacrylamide, 10 mM HEPES, 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 6% glycerol, and was prerun at 10 mA for 30 min in electrophoresis buffer (10 mM HEPES, pH 8, 0.1 mM EDTA). After electrophoresis for 3 h, the gel was stained with silver³⁰ to reveal both CspB and the DNA. The molar ratios of CspB to DNA are indicated.

TABLE 1 Data collection, phase determination and refinement

Crystal form	Native I	Native II	P ₃ 2 ₁	Au	Pt	P ₄ ₃ 2 ₁ 2 Native
Diffraction data						
Detector/wavelength (Å)	FAST/CuK _α	IP/1.008		IP/1.008	FAST/CuK _α	FAST/CuK _α
Resolution (Å)	12–2.55	12–2.3		12–2.3	12–2.65	12–2.7
Unique reflections	2,993	4,222		4,164	2,761	2,252
Completeness (%)	90.9	97.5		96.2	95.5	94.0
R _{sym} (%)	4.6	5.5		4.8	4.4	3.9
m.f.i.d. (%)	—	—		22.4	11.7	—
MIR analysis (10–2.8 Å)						
F _H /ε				2.1	1.6	
R _c				0.597	0.732	
Structure refinement						
Resolution (Å)		10–2.45				10–2.7
R-factor		0.195				0.220
Observations (F ≥ 1σ(F))		3,496				2,217
r.m.s. Δ bond lengths (Å)		0.014				0.021
r.m.s. Δ bond angles (°)		2.5				4.2

CspB was purified and crystallized in two forms²⁰: Trigonal, P₃2₁, *a* = 59.15 Å, *c* = 46.23 Å, and tetragonal, P₄₃2₁2, *a* = 57.01 Å, *c* = 55.71 Å. Diffraction data were collected using an Enraf–Nonius FAST area detector mounted on a FR571 rotating anode or at the EMBL outstation at DESY (Hamburg) on beamline X31 with an image plate detector. For the structure determination by MIR, the trigonal crystal form was used. Derivatives were obtained by soaking crystals with 2 mM KAu(CN)₂ for 48 h (Au) and with 0.25 mM di-μ-chloro-dichlorobis(ethylene)diplatinum(II) for 24 h (Pt). For crystallographic computations the CCP4 programs²¹ were used with exceptions as indicated. One heavy-atom site per derivative was found by difference Patterson techniques and by direct methods²². Heavy-atom parameters were refined and phases were calculated for the Au derivative (mean figure of merit, 0.720). An electron density map calculated at a resolution of 2.8 Å clearly showed the molecular boundaries as well as a β-sheet with the correct left-handed twist between neighbouring strands, thus confirming the choice of enantiomorph. The map was improved by solvent flattening²³, and an initial model comprising residues 1–65 and well defined side chains was built into the electron density using FRODO²⁴. Refinement by simulated annealing using XPLOR²⁵ decreased the R-factor to 0.318. Experimental phases obtained after solvent flattening were combined with model phases²⁶, the model was rebuilt and additional side chains were included. After simulated-annealing refinement (*R* = 0.271), the resolution was extended to 2.45 Å using native data set 2 and refinement including individual temperature factors was continued until convergence. The current model (no Ramachandran outliers) comprises all residues from Met 1 to Ala 67 and 39 H₂O molecules omitting 4 presumably disordered side chains of residues Glu 3, Glu 21, Glu 36 and Glu 66. The tetragonal crystal form of CspB was solved by molecular replacement (XPLOR) using the coordinates of the trigonal crystal form. The cross rotation search followed by Patterson correlation refinement clearly identified one solution. The translation function in space group P₄₃2₁2 yielded a solution which was 11.3σ above the mean (the next highest peak was 9.1σ above the mean) and had an *R*-factor of 0.426 for all data between 8–3.5 Å which reduced to 0.346 after 20 cycles of rigid-body refinement. Subsequent refinement by simulated annealing followed by refinement of individual temperature factors resulted in the current model for this crystal form, which has an *R*-value of 0.220 for all data from 10–2.7 Å without inclusion of solvent molecules. Atomic coordinates and structure amplitudes for both crystal forms have been submitted to the Brookhaven Protein Data Bank. IP, image plate; *R*_{sym}, $\sum \sum |I_i - \langle I_i \rangle| / \sum \sum I_i$, where *I_i* are the individual measurements contributing to the mean reflection intensity $\langle I_i \rangle$; m.f.i.d., $\sum |F_H - F_P| / \sum F_P$, the mean fractional isomorphous difference, where *F_P* and *F_{PH}* are the protein and derivative structure amplitudes, respectively; MIR, multiple isomorphous replacement; *F_H*, heavy atom structure amplitude; ε, residual lack of closure; *R_c*, $\sum |F_{PH} \pm F_P| - F_{H(calc)} / \sum |F_{PH} - F_P|$, where *F_{H(calc)}* is the calculated heavy-atom structure amplitude; *R*-factor, $\sum |F(obs) - F(calc)| / \sum F(obs)$, the crystallographic residual; r.m.s. Δ, root-mean-square from target values.

as the two single strands of a fragment from the *E. coli gyrA* promoter centred about a CCAAT site necessary for binding of *E. coli* CS7.4 (ref. 5) (Fig. 3d). Under our conditions, a fivefold molar excess of CspB over the single strand containing the CCAAT (minus strand) is sufficient to shift quantitatively the band corresponding to free DNA. In contrast, a 20-fold molar excess of CspB does not completely remove the bands resulting

from the free plus strand or from the free double strand. The origin of the apparent sequence or secondary structure preference in the DNA binding of CspB is not understood. The evidence at present supports the view that the CspB protein is likely to be involved in the binding of single-stranded nucleic acids. □

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- Jones, P. G., VanBogelen, R. A. & Neidhardt, F. C. *J. Bact.* **169**, 2092–2095 (1987).
- Goldstein, J., Pollitt, N. S. & Inouye, M. *Proc. natn. Acad. Sci. U.S.A.* **87**, 283–287 (1990).
- Willmsky, G., Bang, H., Fischer, G. & Marahiel, M. A. *J. Bact.* **174**, 6326–6335 (1992).
- La Teana, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10907–10911 (1991).
- Jones, P. G., Krah, R., Tafuri, S. A. & Wolffe, A. P., *J. Bact.* **174**, 5798–5802 (1992).
- Wistow, G. *Nature* **344**, 823–824 (1990).
- Wolffe, A. P., Tafuri, S., Ranjan, M. & Familiari, M. *New Biol.* **4**, 290–298 (1992).
- Landsman, D. *Nucleic Acids Res.* **20**, 2861–2864 (1992).
- Richardson, J. S. *Adv. Protein Chem.* **34**, 167–339 (1981).
- Schnuchel, A. et al. *Nature* (this issue).
- Nagai, K., Oubridge, C., Jessen, T. H., Li, J. & Evans, P. R. *Nature* **348**, 515–520 (1990).
- Crawford, D. R. & Richter, J. D. *Development* **101**, 741–749 (1987).
- Murray, M. T., Krohne, G. & Franke, W. W. *J. Cell Biol.* **112**, 1–11 (1991).
- Murzin, A. G. *EMBO J.* **12**, 861–867 (1993).
- Hynes, T. R. & Fox, R. O. *Proteins: Struct. Funct. Genet.* **10**, 92–105 (1991).
- Brayer, G. D. & McPherson, A. *J. molec. Biol.* **169**, 565–596 (1983).
- Folkers, P. J. M. et al. *Eur. J. Biochem.* **202**, 349–360 (1991).
- Merrill, B. M., Stone, K. L., Cobiainchi, F., Wilson, S. H. & Williams, K. R. *J. biol. Chem.* **263**, 3307–3313 (1988).
- Brennan, C. A. & Platt, T. *J. biol. Chem.* **266**, 17296–17305 (1991).
- Shindelin, H., Herrier, M., Willmsky, G., Marahiel, M. A. & Heinemann, U. *Proteins Struct. Funct. Genet.* **14**, 120–124 (1992).
- CCP4 (The SERC Collaborative Computing Project No. 4, SERC Daresbury Laboratory, Warrington, UK, 1979).
- Sheldrick, G. M. in *Crystallographic Computing* (eds Sheldrick, G. M., Krüger, C. & Goddard, R.) 175–189 (Oxford Univ. Press, Oxford, UK, 1985).
- Wang, B. C. *Meth. Enzym.* **115**, 90–112 (1985).
- Jones, T. A. *J. appl. Crystallogr.* **11**, 268–272 (1978).
- Brünger, A. T., Kuriyan, J. & Karplus, M. *Science* **235**, 458–460 (1987).
- Read, R. *Acta crystallogr.* **A42**, 140–149 (1986).
- Kraulis, J. P. *J. appl. Crystallogr.* **24**, 946–950 (1991).
- Kabsch, W. & Sander, C. *Biopolymers* **22**, 2577–2637 (1983).
- Fried, M. & Crothers, D. M. *Nucleic Acids Res.* **9**, 6505–6525 (1981).
- Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. *Science* **211**, 1437–1438 (1980).

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Structure in solution of the major cold-shock protein from *Bacillus subtilis*

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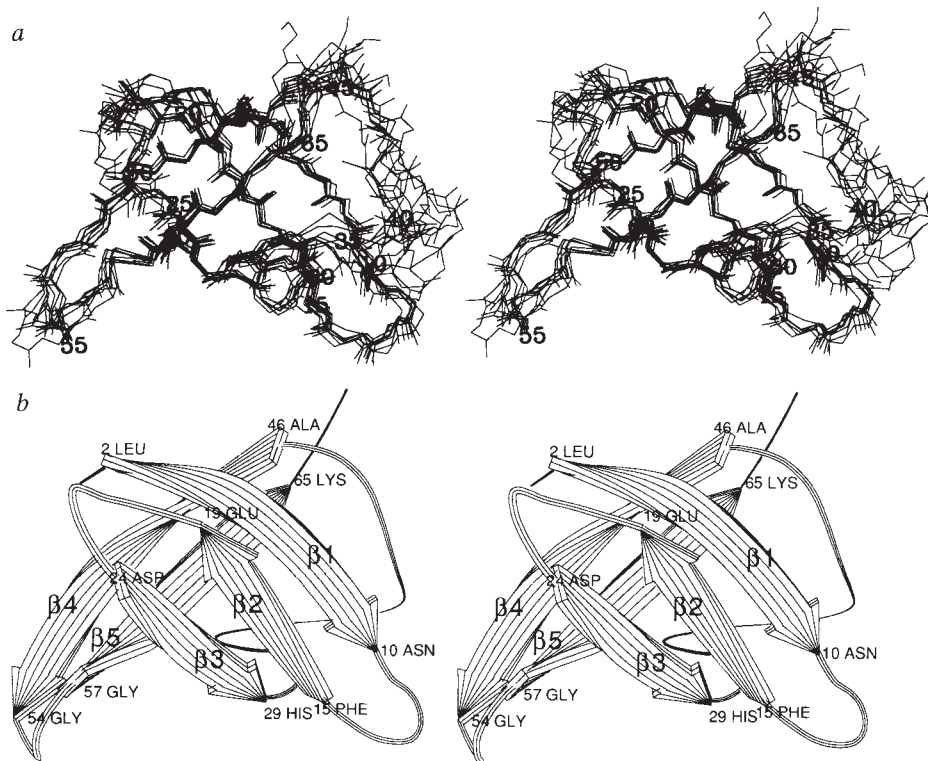
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THE cold-shock domain (CSD) is found in many eukaryotic transcriptional factors and is responsible for the specific binding to DNA of a *cis*-element called the Y-box¹⁻³. The same domain exists in the sequence of the *Xenopus* RNA-binding proteins FRG Y₁ and FRG Y₂ (refs 1, 3). The major cold-shock proteins of *Escherichia coli* (CS7.4)⁴⁻⁷ and *B. subtilis* (CspB)⁸ have sequences that are more than 40 per cent identical to the cold-shock domain. We present here the three-dimensional structure of CspB determined by nuclear magnetic resonance spectroscopy. The 67-residue protein consists of an antiparallel five-stranded β -barrel with

strands connected by turns and loops. The structure resembles that of staphylococcal nuclease and the gene-5 single-stranded-DNA-binding protein⁹⁻¹¹. A three-stranded β -sheet, which contains the conserved RNA-binding motif RNP1 as well as a motif similar to RNP2 in two neighbouring antiparallel β -strands¹²⁻¹⁴, has basic and aromatic residues at its surface which could serve as a binding site for single-stranded DNA. CspB binds to single-stranded DNA in gel retardation experiments.

CspB was expressed in *E. coli* and purified as described⁸. The nuclear magnetic resonance (NMR) structure of CspB was determined using samples that were 1–1.3 mM in protein and 50–60 mM in sodium phosphate at pH 6.0, 6.5 and 7.2 and proton two-dimensional NMR methods¹⁵. The two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) spectra at all pHs were very similar, indicating that the structure of the protein was unaltered within the studied pH range. Resonances of backbone protons for all residues and resonances of side-chain protons for most of the residues were assigned, except for residue 36 (see later). The basic protocol for the calculations has been described¹⁶, and was used with minor modifications which included simulation of two-dimensional NOESY spectra to check whether NOE peaks calculated from NMR structures match experimental NOESY spectra. A total of 20 structures were calculated by a hybrid distance geometry-simulated annealing method¹⁶.

FIG. 1 a, Stereoview of the backbone atoms (N, C α , C, O) of CspB structures best fitted to N, C α and C atoms of all residues except residues 1, 21–24, 34–45, 55–56, 66–67. For clarity, 8 structures were randomly chosen out of 20. b, Ribbon drawing of CspB. The structures were determined from proton–proton distance constraints obtained from NOESY spectra in H₂O and D₂O (pH 7.2) at mixing times of 100 and 80 ms, respectively. The intensities of the NOE crosspeaks were determined from volume integrals and converted into distance constraints as described¹⁶. The intrasidue contacts were not used in the calculations and the total number of inter-residue distance constraints was 435. A complete list of NOE interproton, hydrogen-bonding distance constraints and torsion angle constraints, as well as coordinates of the structures are available from the authors. Simulated annealing calculations were carried out with the program X-PLOR²¹. Distance geometry calculations were carried out with the program DISGEO²². The distance constraint data were supplemented with 35 backbone ϕ torsion angle constraints derived from the ³J_{HNA} coupling constant data and by 21 χ ₁ angles with bounds $\pm 60^\circ$ derived from the NOE and ³J_{AB} data²³. The ϕ constraints ($-130^\circ \pm 60^\circ$) were introduced corresponding to ³J_{HNA} coupling constants larger than 8 Hz²⁴⁻²⁵. Coupling constants ³J_{HNA} were estimated from splittings in the NH-C α H crosspeaks in DQF COSY spectra²⁶, whereas ³J_{AB} coupling constants were from the E.COSY spectrum²⁷. 113 non-NOE distance constraints were also present in the calculations¹⁶. For the final refinement, the NOE tables were supplemented by 22 constraints for the 11 hydrogen bonds identified on the basis of the examination of the structures during the secondary structure analysis. All protons were explicitly defined in the dynamical simulated annealing calculations, although in some cases additional terms were added to the upper bounds that correspond to the pseudatom correction²⁸. The distance bounds of the distance constraints were set



to $d \pm 0.5 \text{ \AA}$ for the distance constraints between 2.2–3.3 $\text{\AA}$, and to $d - 0.6 \text{ \AA} / + 0.8 \text{ \AA}$ for distance constraints 3.4–4.2 $\text{\AA}$. The linewidths of peaks in NMR spectra of CspB at pH 6.0 and 6.5 were somewhat larger than expected for a monomeric 7.4K protein such as CspB, indicative of some aggregation in solution (below pH 5.6 the protein precipitates). As G5BP formed a symmetrical dimer, dimerization of CspB was taken into account during structure calculations. After completion of sequential resonance assignments, in which no NOEs inconsistent with the notion of a β -strand structure were detected, it was clear that few, if any, intermolecular NOEs would be present. Nevertheless, an iterative approach in the structure calculation was used by carrying out a series of calculations with more and more constraints incorporated at each successive stage. No conflicting NOEs were noted at the final stage and it was therefore concluded that all NOEs were intramolecular.

CspB consists entirely of β -strands connected by turns and loops (Fig. 1). Superposition of the final structures is shown in Fig. 1a. All simulated annealing structures satisfy the experimental constraints (there were no residual constraint violations (r.m.s.d.) larger than 0.5 Å for all 435 distance constraints), show very small deviations from idealized covalent geometry, and have negative Lennard-Jones van der Waals energies ($F_{1-2} = -354 \pm 20$ kcal mol⁻¹). The backbone of CspB within β -strands is well determined. The segment of residues from 35 to 41 shows the greatest variability in positions of atoms (Fig. 1a). This variability may be due in part to paucity of NOEs in most of the residues in this fragment. For example, the assignment of Glu 36 in this segment could not be made because of the lack of any NOE peaks for this residue. As Glu 36 also exhibits the highest surface accessibility (120 Å², compared to the average in the molecule of 20 Å² per residue), the NMR data suggest an increased flexibility of the fragment around this residue in the protein. The average of the r.m.s. differences for the backbone atoms in β -strands among the structures were 0.65 ± 0.12 Å and 1.38 ± 0.15 Å for all atoms. The corresponding r.m.s. difference averages for heavy atoms in the loops were 2.2 ± 0.4 Å for the backbone atoms and 3.9 ± 0.7 Å for all atoms.

Five well defined β -strands (strands β_1 , β_2 , β_3 , β_4 and β_5) were identified between residues 2–10, 15–19, 24–29, 46–54 and 57–65, respectively (Fig. 1b). Bulges are located at residues 7 and 62 in β_1 and β_5 , respectively. The five strands form two β -sheets, each sheet consists of three strands, with strand β_1 shared by the two sheets. The antiparallel β -sheet 1 is composed of strands β_1 (residues 7–10), β_2 , β_3 , and the antiparallel β -sheet 2 of strands β_1 (residues 2–5), β_4 , and β_5 . The two β -sheets make up a β -barrel with hydrophobic residues: Leu 2, Val 6, Phe 9, Ile 18, Val 20, Val 26, Val 28, Leu 41, Val 47, Phe 49, Ile 51, and Val 63 located inside the barrel.

Strand β_2 contains the RNPI sequence motif found in a variety of RNA-binding proteins and also identified in the CSD

domain¹⁴. Superposition of the backbone of the RNPI motif of U1 snRNP-A, for which the three-dimensional structure is known^{12,14}, on CspB shows that the conformation has been conserved of the RNA-binding domains RNPI and, to a lesser extent, RNP2 in CspB. For RNPI, residues Lys 13, Gly 14, Phe 15, Gly 16, Phe 17 and Ile 18 of CspB are structurally equivalent to Arg 52, Gly 53, Glu 54, Ala 55, Phe 56 and Val 37 of U1 snRNP-A, respectively. Matching is not as extensive for the RNP2 motif: CspBs Val 26, Phe 27 and Val 28 correspond to Ile 12, Tyr 13 and Ile 14 of U1 snRNP-A. The RNA-binding motifs in U1 snRNP-A (ref. 12) and in CspB are located on two neighbouring antiparallel β -strands which are sequential in CspB but not in U1 snRNP-A.

A search for structural similarities to known protein folds using the program WHAT IF¹⁷ revealed that the β -barrel structure of CspB is similar to those of the OB-fold proteins⁹ (Fig. 2a), the gene-5 single-stranded-DNA-binding protein (G5BP)¹¹, and yeast inorganic pyrophosphatase¹⁸. It was suggested that the single-stranded-DNA-binding site is located at a surface patch rich in aromatic and basic side chains in the G5BP molecule¹¹. A similar concentration of aromatic residues is found in the β -sheet 1 of CspB which is structurally similar to the patch of G5BP. A cluster of seven aromatic residues from the total of 9 in CspB is located in β -sheet 1. Six of them (Trp 8, Phe 15, Phe 17, Phe 27, His 29 and Phe 30) are on the one side of the β -sheet that is exposed to solvent (Fig. 2b). The proposed binding site also roughly corresponds to the common oligonucleotide-binding site of the OB-fold proteins⁹.

Gel retardation experiments were therefore carried out to assay DNA binding to CspB (Fig. 3). For these experiments we used two different single-stranded DNA oligomers. The first oligomer was 35 bases long and contained one reverse complement CCAAT-box (ATTGG) necessary for DNA binding of the CS7.4 protein⁷. The second DNA probe was a 54-nucleotide oligomer which was used in the FRG Y₁ and FRG Y₂ mobility

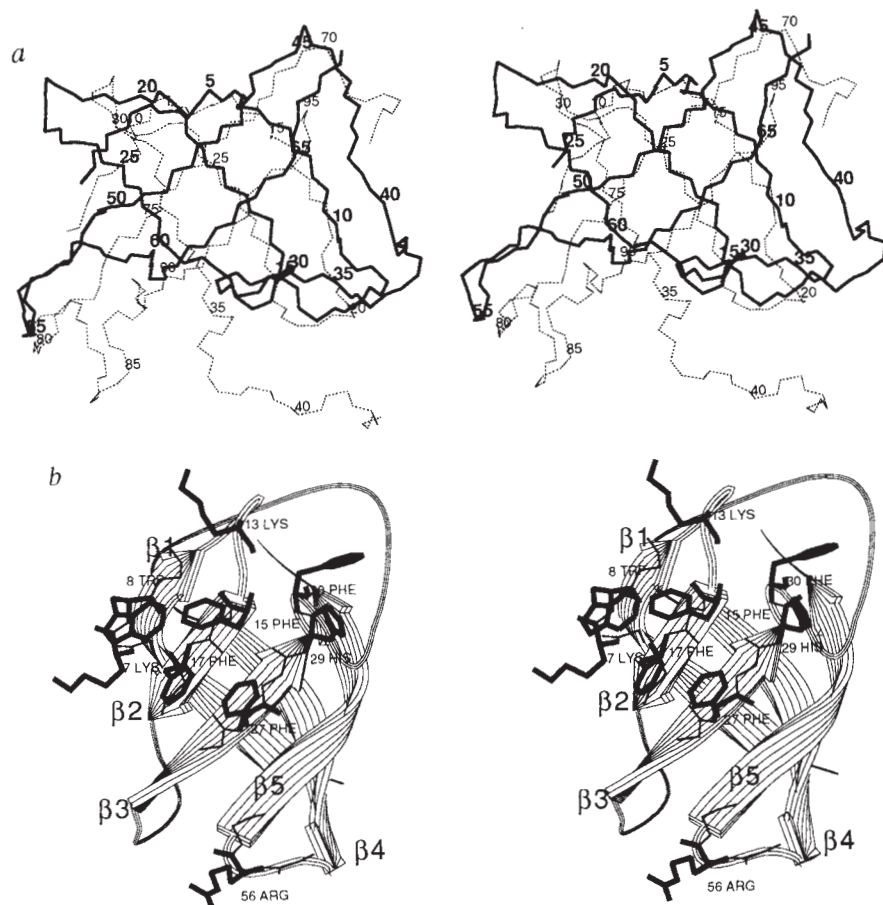


FIG. 2 a, Superposition of the C α tracing of CspB (solid line) and staphylococcal nuclease (broken line). b, Stereopicture of CspB with the side chain of Arg 56 and side chains of basic and aromatic residues in β -sheet 1 (except Phe 9).

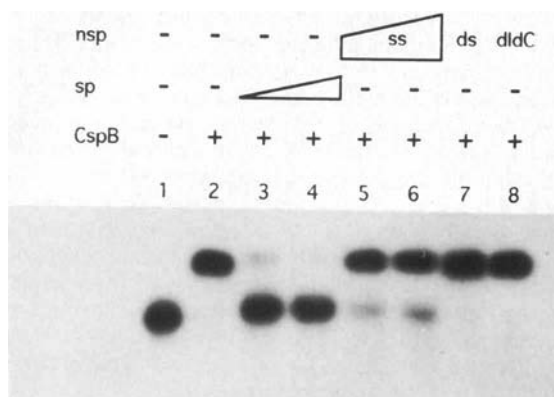


FIG. 3 Gel retardation of the 54-mer oligonucleotide (sp) GAATTCGCA-GACGTGGGAATCCTACTGATTGGCCAAGGTGCTGGTGGTGTGG by CspB. The 54-mer oligonucleotide was end-labelled with [γ - 32 P] dATP (110 TBq mmol $^{-1}$) using T4 polynucleotide kinase. Constant amounts of the labelled oligonucleotide (1.25 pmol) were incubated with various amounts of the purified CspB protein in 10 μ l of a buffer containing 20 mM Tris-HCl, pH 8.6, 50 mM NaCl, 5 mM MgCl $_2$, 5% glycerol for 30 min at 25 $^{\circ}$ C. Then 5 μ l of loading dye buffer (10% glycerol, 0.1% bromophenol blue in 1x TBE, Tris, borate, EDTA) were added and samples were loaded on non-denaturing 12.5% polyacrylamide gels. After electrophoresis gels were subjected to autoradiography at -80 $^{\circ}$ C. The concentrations of CspB and non-labelled oligonucleotide were as follows: lane 1, no protein; lane 2, 25 pmol CspB; lane 3, 25 pmol CspB and 25 pmol specific 54-mer oligonucleotide (sp); lane 4, 25 pmol CspB and 50 pmol specific 54-mer oligonucleotide (sp); lane 5, 25 pmol CspB and 50 pmol nonspecific 54-mer oligonucleotide (nsp/ss); lane 6, 25 pmol CspB and 75 pmol nonspecific 54-mer oligonucleotide (nsp/ss); lane 7, 25 pmol CspB and 0.5 pmol plasmid pTZ18U (2,860 bp, USB Corp., Cleveland); lane 8, 25 pmol CspB and 1 μ g poly(dI-dC). The sequence of the nonspecific 54-mer oligonucleotide (nsp/ss) is: CAT-GCTGCGTGAACAGGTTGCTCAGCTGAAACAGAAAGTTGGTGAACGTTGATA.

shift experiments¹⁹. It contained only a single copy of the ATTGG box embedded in the consensus sequence CTG on the 3' side and CCAA on the 5' side. Both oligomers bound to CspB. In similar experiments double-stranded DNA did not bind to CspB (Fig. 3).

As CspB is a highly acidic protein with a *pI* of 4.3, it must overcome the charge repulsion of a negatively charged DNA in order to form a DNA-protein complex. It can be seen from Fig. 2b that there is a cluster of basic residues (Lys 7, Lys 13, His 29 and Arg 56, from a total of seven basic residues) on a solvent-accessible face of the molecule. These residues, together with the aromatic residues already mentioned, could serve as a site for DNA binding. Our description of CspB as a nucleic-acid-binding domain which is conserved throughout evolution from bacteria to man is in agreement with that derived independently by X-ray structure analysis²⁰. □

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- Wolffe, A. P., Tafuri, S., Ranjan, M. & Familary, M. *New Biol.* **4**, 290-298 (1992).
- Wistow, G. *Nature* **344**, 823-824 (1990).
- Tafuri, S. R. & Wolffe, A. P. *Proc. natn. Acad. Sci. U.S.A.* **87**, 9028-9032 (1990).
- Jones, P. G., Van Bogelen, R. A. & Neidhardt, F. C. *J. Bact.* **169**, 2092-2095 (1987).
- Jones, P. G., Krah, R., Tafuri, A. A. & Wolffe, A. P. *J. Bact.* **174**, 5798-5802 (1992).
- Goldstein, J., Pollitt, N. S. & Inouye, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 283-287 (1990).
- La Teana, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10907-10911 (1991).
- Willimsky, G., Bang, H., Fischer, G. & Marahiel, M. A. *J. Bact.* **174**, 6326-6335 (1992).
- Murzin, A. G. & Chothia, C. *Curr. Opin. Struct. Biol.* **2**, 895-903 (1992).
- Brayer, G. D. & McPherson, A. *J. molec. Biol.* **169**, 565-596 (1982).
- McPherson, A. & Brayer, G. D. *Biological Macromolecules and Assemblies* **2**, (eds Jumak, F. A. & McPherson, A.) 323-392 (Wiley, New York, 1985).
- Nagai, K., Oubridge, C., Jessen, T. H., Li, J. & Evans, R. *Nature* **348**, 515-520 (1990).
- Hoffman, D. W., Query, C. C., Golden, B. L., White, S. W. & Keene, J. B. *Proc. natn. Acad. Sci. U.S.A.* **88**, 2495-2499 (1991).
- Landsman, D. *Nucleic Acids Res.* **20**, 2861-2864 (1992).
- Wüthrich, K. *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986).
- Holak, T. A., Gondol, D., Otlewski, J. & Wilusz, T. *J. molec. Biol.* **210**, 635-648 (1989).
- Vriend, G. *J. molec. Graphics* **8**, 52-56 (1990).
- Arutyunyan, E. G. et al. *Dokl. Biochem. (Engl. Transl.)* **258**, 189-195 (1981).
- Tafuri, S. R. & Wolffe, A. P. *New Biol.* **4**, 349-359 (1992).

- Schindelin, H., Marahiel, M. A. & Heinemann, U. *Nature* **364**, 164-168 (1993).
- Brünger, A. T. *J. molec. Biol.* **203**, 803-816 (1988).
- Havel, T. F. & Wüthrich, K. *J. molec. Biol.* **182**, 281-294 (1985).
- Wagner, G. et al. *J. molec. Biol.* **196**, 611-639 (1987).
- Pardi, A., Billeter, M. & Wüthrich, K. *J. molec. Biol.* **180**, 741-751 (1984).
- Kline, A., Braun, W. & Wüthrich, K. *J. molec. Biol.* **204**, 657-724 (1988).
- Marion, D. & Wüthrich, K. *Biochem. Biophys. Res. Commun.* **113**, 967-974 (1983).
- Griesinger, C., Sørensen, O. W. & Ernst, R. R. *J. magn. Reson.* **75**, 474-492 (1987).
- Wüthrich, K., Billeter, M. & Braun, W. *J. molec. Biol.* **169**, 949-961 (1983).

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Three-dimensional atomic model of F-actin decorated with *Dictyostellium* myosin S1

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ELUCIDATION of the molecular contacts between actin and myosin is central to understanding the force-generating process in muscle and other cells. Actin, a highly conserved globular protein found in all eukaryotes, polymerizes into filaments (F-actin) for most of its biological functions. Myosins, which are more diverse in sequence, share a conserved globular head of about 900 amino acids in length (subfragment-1 or S1) at the N-terminal end of the molecule. S1 contains all the elements necessary for mechanochemical force transduction *in vitro*^{1,2}. Here we report an atomic model for the actomyosin complex produced by combining the atomic X-ray structure of F-actin^{3,4} and chicken myosin S1⁵ with a three-dimensional reconstruction from electron micrographs of frozen-hydrated F-actin decorated with recombinant *Dictyostellium* myosin S1. The accuracy of the reconstruction shows the position of actin and myosin molecules unambiguously.

We used energy-filtered electron microscopy to visualize frozen-hydrated samples of rabbit F-actin decorated with a recombinant *Dictyostellium* myosin S1 (*Dicty*-S1)⁶. Zero-loss energy filtering improves imaging of frozen-hydrated specimens as it increases the contrast by removal of inelastically scattered electrons⁷, thereby allowing micrographs to be taken close to the electron optical focus, which results in a higher spatial resolution of the image. Moreover, specimens can be observed at a lower electron dose, reducing beam damage. Examples of decorated filaments are shown in Fig. 1a,b. The difference in contrast at different defocus is obvious: high-contrast images (Fig. 1a) recorded at a higher defocus demonstrate the quality of the arrowhead pattern and have been used to check the quality of the decoration, whereas images recorded at lower defocus show the higher structural resolution (Fig. 1b). Power spectra resulting from diffraction analysis of such images revealed structural information content up to high resolution (Fig. 1c, d). We computed a three-dimensional electron-density map with a resolution of about 2.0 nm by means of a helical reconstruction⁸, which included data from 42 arrowheads or 546 single myosin molecules.

The resulting electron density clearly shows the actin monomers forming the filament as well as individual myosin heads

interacting with the actin filament in a tangential manner. Figure 2a shows a corresponding surface representation of the electron density. Using the computer graphics program FRODO⁹ we fitted the F-actin high-resolution structure⁴ directly and determined the relative origin of F-actin with about $\pm 1^\circ$ azimuthal and ± 3 -Å translation precision. The resulting fit of the atomic structure of F-actin to the electron-micrograph reconstruction of *Dicty*-S1-decorated actin is shown in Fig. 2b. The actin model can be placed into the reconstruction without ambiguity. We then docked the atomic model of chicken myosin S1 (ref. 5) onto the model of the actin filament using the full electron-density

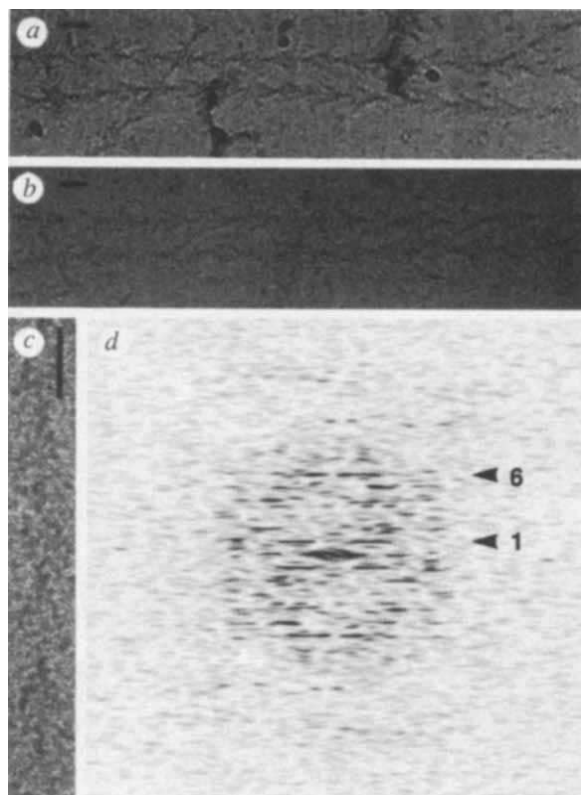


FIG. 1 Images and computed diffraction pattern of decorated actin filaments. *a, b*, Typical images of F-actin filaments decorated with *Dictyostelium* myosin-head fragment (MHF) embedded in vitrified ice. Micrographs taken with an energy filter microscope Zeiss EM902 at 80 kV under low-dose conditions (maximum specimen electron dose $\sim 500 \text{ e}^- \text{ nm}^{-2}$). Magnification $\times 50,000$, energy loss $\Delta E = 0 \text{ eV}$ (zero-loss mode), energy width $\Delta E = 20 \text{ eV}$. Defocus in the range $500 \text{ nm} - 1.5 \mu\text{m}$. The examples shown are *a*, $1,100 \pm 100 \text{ nm}$ and *b*, $750 \pm 100 \text{ nm}$. Comparison of *a* and *b* demonstrates the loss of contrast in images taken closer to focus, which is partly compensated by zero-loss energy filtering⁷. Images as in *a* were used to check the quality of the actomyosin decoration, whereas images as in *b* were included in the reconstruction. Kodak SO163 film was developed in undiluted Kodak D19 for 12 min at 25°C . *c*, Sample of a typical filament area used in the reconstruction. Because of the enhanced contrast from energy filtering, individual S1 molecules forming the arrowhead pattern can be seen clearly. Improved resolution is reflected in the visibility of layer lines in the diffraction pattern calculated from the filament shown in *c*. The central part of this calculated diffraction pattern is shown in *d*. Arrows, layer-line positions in the diffraction pattern showing strong signal at layer lines (1) 1, 2, 4, 5 and 6, as well as weak signal at 3, 7 and 8 (corresponding to a resolution of 4.5 nm). Scale bars, 35.4 nm.

METHODS *Dicty*-S1 was purified as described⁶ and stored as frozen solution (25 mg ml^{-1}) in 30% sucrose (w/v) at -80°C . The final concentration of MHF in the sample was $0.4-0.6 \text{ mg ml}^{-1}$. Actin from rabbit muscle²¹ was mixed with *Dicty*-S1 in a ratio of 1 to 3 (w/w) in decoration buffer (KCl, 50 mM; MgCl_2 , 2 mM; Tris/HCl, 20 mM; ATP, 5 mM; pH 7.3; final concentration). Optimal decoration was observed after incubation for 18–24 h at 4°C . Samples were prepared as thin layers over holes and vitrified by rapid freezing in liquid ethane.

map of *Dicty*-S1-decorated actin as a guide. In the docking process we attempted to optimize the fit of the atomic S1 heavy chain to the external surface rather than the atomic high-resolution interactions of the two molecular models. The overall shape of the S1 head is highly asymmetric and consequently there is only one way to fit the atomic model to the density (see Fig. 3). Multiple attempts at docking yield estimates with a precision of ± 3 Å. The resulting positioning of the two molecules shows that in the contact region (Fig. 3b) the atomic models interpenetrate, indicating that structural changes to S1 must occur during formation of the rigor (ATP-free) complex.

An important element of muscle contraction is the ordering of the binding of myosin to actin: an initial stereoscopic weak binding allows a transition to strong binding, which results in force production. Prominent features of the S1 X-ray structure are the light chains arranged at the C-terminal end and the N-terminal β -barrel that protrudes out of the bulk of the head. In the reconstruction the C-terminal part of the molecule is not seen, which indicates disorder in the distal region of S1 (see Fig. 3b). Note that the N-terminal β -barrel lies within the density that connects adjacent heads, resulting in the juxtaposition of residues in neighbouring heads that can be crosslinked with a zero-length crosslinker¹¹, providing independent evidence supporting the model. The slight miss-set of this domain relative to the density of the reconstruction may arise from a miss-orientation of the β -barrel caused by crystal packing into the crystalline lattice of chicken myosin S1.

The interaction of myosin and actin derived here is very similar to that observed for the fit of the atomic models to rabbit chymotryptic S1-decorated actin¹⁰. The model building reveals three regions of close contact between a single actin monomer and the myosin head (Fig. 4a). The first contact involves a highly charged lysine-rich loop on myosin (amino acids 626 to 647; chicken sequences¹²) which can interact with known myosin-binding residues of rabbit actin including Asp 1, Glu 2, Glu 3, Asp 4, Asp 24, Asp 25, Glu 99 and Glu 100. This contact is nonspecific electrostatic and may be responsible for the ionic-strength-dependent 'weak' interaction between actin and myosin¹³. Pressure-jump studies¹⁴ indicate that a significant volume change accompanies the weak-strong transition thought to result in force generation. Therefore, it seems likely that this volume change arises from an interaction between the charged-loop and the actin N terminus and that this interaction plays a role in the subsequent development of the rigor interaction.

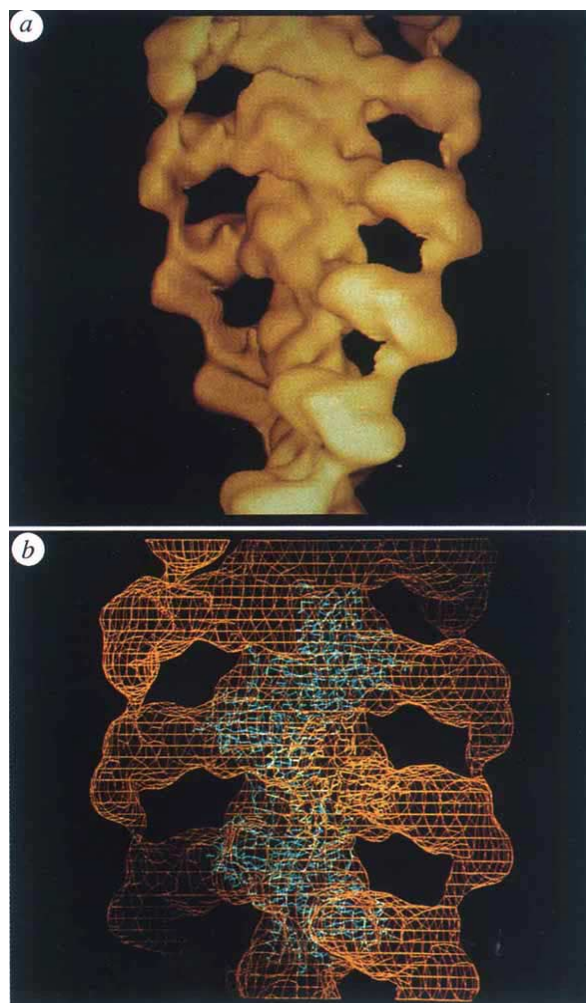
The second contact is made by the segment defined by Pro 529 to Lys 553 of the myosin heavy chain. This segment corresponds to a broken helix on the surface of S1. The resulting interaction is potentially stereospecific as it involves exposed hydrophobic residues on the surface of both actin (Ala 144, Ile 341, Ile 345, Leu 349 and Phe 352) and myosin (Pro 529, Met 530, Ile 535, Met 541, Phe 542 and Pro 543). The distribution of hydrophobic and charged residues within this segment follows an identical pattern in all known myosin sequences, and the corresponding actin regions (residues 144–148 and 339–354) are also completely conserved.

The third contact involves the loop formed by residues 403–416 of myosin (Fig. 4a) and the region around an exposed pair of proline residues (332–333) of actin. The functional importance of this region of the myosin molecule is demonstrated by the mutation of a conserved arginine residue (405) to glutamine in this loop, which is one cause of familial hypertrophic cardiomyopathy¹⁵. Further, the phosphorylation site that regulates the actin-activated Mg^{2+} -ATPase activity of *Acanthamoeba* myosin 1A, 1B and 1C is in this region¹⁶.

These three contacts involve a single actin subunit and define the primary site of actomyosin interaction. In addition, the model building brings a second region of the myosin heavy chain into the proximity of the neighbouring actin monomer one actin helix turn below. The main contribution to this secondary interaction with actin comes from a loop that corresponds to

FIG. 2 Three-dimensional map of *Dictyostelium* S1-decorated rabbit F-actin. *a*, Surface model of the electron density; *b*, grid model of the electron density.

METHODS. The atomic model of F-actin⁴ was fitted into the electron density as described in the text. Image processing and helical reconstruction was performed using programs specially developed for a Convex C1 and a SG Indigo. Helical reconstructions of individual filament areas were calculated as described⁸ and examined individually with a graphics display. Filaments were selected for averaging on the basis of the image quality of the original filament region and common features of the calculated three-dimensional maps. Only straight filament regions of identical periodicity were included, thus selecting for filaments with identical orientation in the ice layer and avoiding the need for unbending or tilt corrections. The filament areas used covered 5 to 9 arrowheads. As demonstrated in Fig. 1*c, d*, such filament areas, although small, give good diffraction patterns with well-defined layer lines. The phase origins of individual filaments (relative rotational (azimuthal) and translation origins), were determined before averaging filaments. Our data are consistent with a 13/6 F-actin symmetry. Averaging filaments imaged in an energy filter transition electron microscope at varying defocus does not necessarily improve final resolution, so we first corrected for errors in instrumentation as modelled in the contrast transfer function (CTF). To avoid systematic errors arising from incorrect parameters in the correction for differing CTFs, the reconstruction presented here consists of the average of filaments from just one, typical micrograph, which overall showed the best diffraction patterns of filaments. This micrograph was imaged close to focus and showed good contrast in thin ice. The resulting averaged map is shown, after correction for the effects of the CTF which is particularly important for determining the radial mass distribution, as has been shown for tobacco mosaic virus (TMV)²². Defocus parameter and magnification were determined directly on an optical bench by observing the Thon rings. The corrected map depends only to a small extent on the assumed error in defocus but to a large extent on variation in the ratio *R* of phase contrast and amplitude contrast. As the equator of the diffraction pattern is very sensitive to *R*, varying *R* influences the radial density profile of the final map. Furthermore, the CTF correction affects the intensity ratios of different layer lines²³ and thus the general appearance of the CTF-corrected density map. A value of *R*=0.15 gave a radial density distribution similar to that expected from the atomic F-actin model together with the molecular masses of actin and myosin S1. As this *R* value is very similar to the *R* value of 0.14 determined for TMV particles²², *R*=0.14 was used for correcting the map as shown. This map includes data of 42 arrowheads, or 546 single myosin molecules. The resolution is restricted to 2 nm in axial and radial directions.



a Primary actin binding site:

Region 1

	630	640	650
chicken	FATYGGAEAGGGG	KKGGKKGGSS	FQTVSALFRENL
Dicty	FN...DPNIAS	·RA·KKGANFITVAAQYKEQL	

Region 2

	530	540	550
chicken	LIE·K·PMGIFSILEECMF	PKATDTSFKNKLYDQHL	
Dicty	LIDGRQPPGILALLDEQSVFP	NATDNTLITKLHSHFS	

Region 3

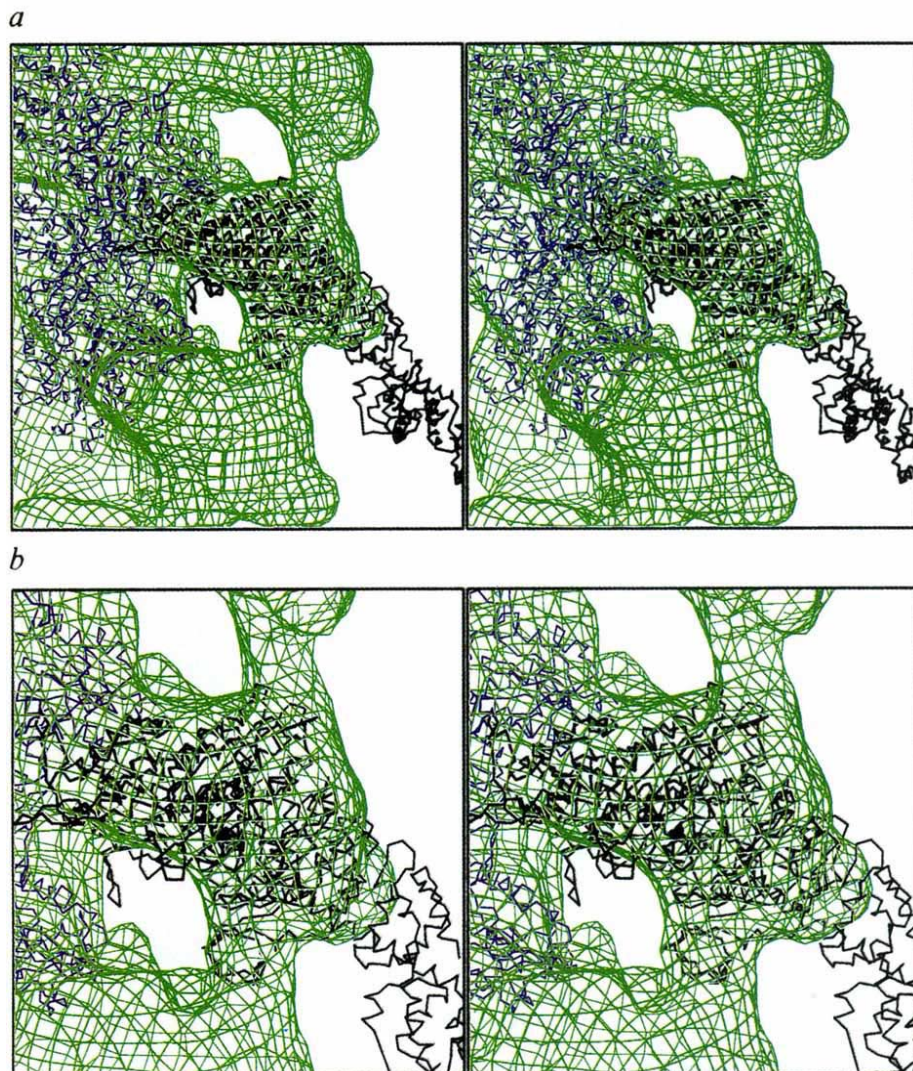
	400	410
chicken	LLKALCYP	PRVKVGNFVTKG
Dicty	LEKALMEPRILAGRD	LVAQH

b Secondary actin binding site:

	560	570	580
chicken	GKSNFQ	KPKAKGAEAHFSLVHYAGTV	
Dicty	KKNAKYEEPRF	·SKTE·FGVTHYAGQV	

FIG. 4 Alignment of chicken myosin and *Dictyostelium* myosin amino-acid residues participating in the rigor state interaction with actin. *a*, The primary binding site is formed by three regions. Region 1 corresponds to the highly charged lysine-rich loop that connects the 50K and 20K domains. This region is disordered in the three-dimensional structure and the corresponding residues (626–647) are therefore missing in the atomic model of S1 used for the docking. However, the 20 unresolved residues would be well positioned to interact with negatively charged residues of actin. Region 2 involves residues 529–553 located in the lower 50K domain. This segment is part of a broken helix, the first part of which extends from Gly 516 to Phe 542 and contains a bulge at Pro 529. The second part of the helix is formed by residues Asp 547 to His 558. Region 3 involves a loop formed by residues 403–416 of the upper 50K domain. *b*, The main contribution to a potential secondary actomyosin interaction comes from a loop formed by residues 567–578. The alignment shows the generally high degree of functional conservation between actin binding residues of myosins from two widely separated phyla. Positively charged residues are shown in red, negatively charged residues in blue; numbering according to ref. 12.

FIG. 3 Stereo view of the optimized fit of the atomic models of S1 and F-actin in the molecular envelope of *Dicty-S1*-decorated actin (a) and enlarged view showing the interaction between the myosin head and actin (b). The electron-density map derived from a three-dimensional reconstruction of electron micrographs of *Dicty-S1*-decorated actin is shown as squirrel-cage (green). Actin monomers (blue) and one S1 molecule (black) are shown as $C\alpha$ models. Fitting of the molecular models is described in the text. Note that the protruding N-terminal β -barrel lies essentially within the density that connects adjacent heads. The light-chain binding region projects away from the filament axis and lies completely outside the electron density. This indicates disorder in the distal part of S1 — flexibility of this region might be of functional importance. At the proximal end, S1 is in contact range with two actin monomers. Residues from both the upper and lower 50K domains of S1 (ref. 5) interact with one of the actin monomers to form the primary binding site that comprises most of the surface area of the interaction. The main contribution to a possible secondary interaction comes from a loop that can be seen extending underneath the primary binding site towards the neighbouring actin subunit. Details of the interaction are described in the text.



residues 567–578 in the chicken myosin sequence¹² (Fig. 3). This loop projects underneath the major actin binding site towards the C-terminus of the α -helix formed by residues Trp 79 to Asn 92 of actin. This region shows the most significant differences between our reconstruction and that obtained using chymotryptic S1 (ref. 17). This finding may indeed reflect a genuine structural difference between *Dictyostelium* and vertebrate myosins, as this segment of the myosin heavy chain displays some of the most pronounced differences between *Dictyostelium* and vertebrate sequences in the actomyosin interface (Fig. 4b).

Remarkable functional conservation is found in the way in which actin and myosin isoforms from different species interact. For example, every heterologous mixture of actin and myosin tested so far shows functional interaction. Both *Dictyostelium*

actin and skeletal muscle actin move at about $2 \mu\text{m s}^{-1}$ along *Dictyostelium* myosin, and both forms of actin move at about $5 \mu\text{m s}^{-1}$ along skeletal muscle myosin¹⁸. The concentration of rabbit actin required for half-maximal activation of the myosin ATPase activity (K_{app}) is similar for *Dictyostelium* and vertebrate myosin¹⁹, and *Dictyostelium* myosin has an affinity for rabbit actin indistinguishable from that of rabbit myosin²⁰. Such findings suggest a constancy of structure of the actomyosin complex and of the details of the molecular contacts at the actomyosin interface. The present study of the actomyosin interaction in two widely separated phyla confirms a high degree of conservation in structure and sequence at the working interface, and supports the mechanistic model for the cross-bridge cycle proposed elsewhere¹⁰. □

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- Toyoshima, Y. Y. *et al.* *Nature* **328**, 536–539 (1987).
- Kishino, A. & Yanagida, T. *Nature* **334**, 74–76 (1988).
- Holmes, K. C., Popp, D., Gebhard, W. & Kabsch, W. *Nature* **347**, 44–49 (1990).
- Lorenz, M., Rypniewski, W. R., Popp, D. & Holmes, K. C. *J. molec. Biol.* (in the press).
- Rayment, I. *et al.* *Science* (in the press).
- Manstein, D. J., Ruppel, K. M. & Spudich, J. A. *Science* **246**, 656–658 (1989).
- Schröder, R. R., Hofmann, W. & Menetret, J.-F. *J. struct. Biol.* **105**, 28–34 (1990).
- Moore, P. B., Huxley, H. E. & DeRosier, D. J. *J. molec. Biol.* **50**, 279–295 (1970).
- Jones, T. A. *Meth. Enzym.* **115**, 157–171 (1985).
- Rayment, I. *et al.* *Science* (in the press).
- Onishi, H., Maita, T., Matsuda, G. & Fujiwara, K. *J. biol. chem.* **265**, 19362–19368 (1990).
- Maita, T. *et al.* *J. Biochem., Tokyo* **110**, 75–87 (1991).
- Brenner, B., Schoenberg, M., Chalovich, J. M., Greene, L. E. & Eisenberg, E. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7288–7291 (1982).
- Geeves, M. A. *Phil. Trans. R. Soc. Lond.* **B336**, 63–70 (1992).

- Geisterfer-Lowrance, A. T. *et al.* *Cell* **62**, 999–1006 (1990).
- Brzeska, H., Lynch, T. J., Martin, B., Corigliano-Murphy, A. & Korn, E. D. *J. biol. Chem.* **265**, 16138–16144 (1990).
- Milligan, R. A., Whittaker, M. & Safer, D. *Nature* **348**, 217–221 (1990).
- Kron, S. J. & Spudich, J. A. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6272–6276 (1988).
- Truong, T., Medley, Q. G. & Côté, G. P. *J. biol. Chem.* **267**, 9767–9772 (1992).
- Ritchie, M. D., Geeves, M. A., Woodward, S. K. A. & Manstein, D. J. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Spudich, J. A. & Watt, S. J. *J. biol. Chem.* **246**, 4866–4871 (1971).
- Smith, M. F. & Langmore, J. P. *J. molec. Biol.* **226**, 763–774 (1992).
- Schröder, R. R., Manstein, D., Jahn, W. & Spudich, J. A. *Proc. 51st MSA Conf., Cincinnati* (in the press).

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